

05-31-00

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05/30/00  
JC840 U.S. PTO

Practitioner's Docket No. 701039-48802 C

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

JC531 U.S. PTO  
09/580803  
05/30/00

Box Patent Application  
Assistant Commissioner for Patents  
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of  
Inventor(s): Michael KLAGSBRUN, Shay SOKER, Hua-Quan MIAO, Seiji TAKASHIMA.

**WARNING:** 37 C.F.R. § 1.41(a)(1) points out:

*"(a) A patent is applied for in the name or names of the actual inventor or inventors.*

*(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."*

For (title): ANTAGONISTS OF NEUROPILIN RECEPTOR FUNCTION AND USE THEREOF

CERTIFICATION UNDER 37 C.F.R. 1.10\*

(Express Mail label number is **mandatory**.)

(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 30 May 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EK571074376US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Nicholas A. Zachariades

(type or print name of person mailing paper)

*Nicholas A. Zachariades*

Signature of person mailing paper

**WARNING:** Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

**\*WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(b).  
"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

(Application Transmittal—page 1 of 12)

09580803 053000

## 1. Type of Application

This new application is for a(n)

(check one applicable item below)

- ☒ Original (nonprovisional)  
☐ Design  
☐ Plant

**WARNING:** Do not use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.

**WARNING:** Do not use this transmittal for the filing of a provisional application.

**NOTE:** If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

- ☐ Divisional.  
☒ Continuation.  
☐ Continuation-in-part (C-I-P).

## 2. Benefit of Prior U.S. Application(s) (35 U.S.C. 119(e), 120, or 121)

**NOTE:** A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

**NOTE** If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

**WARNING:** If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121

[illegible]

☒ The new application being transmitted claims the benefit of prior U.S. application(s).  
Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE  
BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

**A. Required for Filing Date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application**

**WARNING:** *DO NOT* submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. 1.84, see Notice of March 9, 1988. (1990 O.G. 57-62).

*NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page. . ." 37 C.F.R. § 1.84(c)).*

(complete the following, if applicable)

[ ] The enclosed drawing(s) are photograph(s), and there is also attached a “PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S).” 37 C.F.R. § 1.84(b).

[ ]	Formal
[X]	Informal

_____	Pages of declaration and power of attorney
<u>1</u>	Pages of Abstract
9	Other (Sequence Listing)

4. **Additional Papers Enclosed**

- ☐ Amendment to claims
- ☐ Cancel in this applications claims \_\_\_\_\_ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
- ☐ Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)
- ☐ Preliminary Amendment
- ☐ Information Disclosure Statement (37 C.F.R. § 1.98)
- ☐ Form PTO-1449 (PTO/SB/08A and 08B)
- ☐ Citations
- ☐ Declaration of Biological Deposit
- ☐ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.
- ☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative
- ☐ Special Comments
- ☐ Other

5. **Declaration or Oath (including power of attorney)**

*NOTE: A newly executed declaration is not required in a continuation or divisional application provided the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47 then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. § 1.63(d)(1)-(3).*

*NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and the residence, post office address and country of citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).*

☐ Enclosed

Executed by

(check all applicable boxes)

- ☐ inventor(s).
- ☐ legal representative of inventor(s). 37 C.F.R. § 1.42 or 1.43.
- ☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

☒ Not Enclosed.

**NOTE:** *Where the filing is a completion in the U.S. of an International Application, or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.*

☐ Application is made by a person authorized under 37 C.F.R. 1.41(c) on behalf of *all* the above named inventor(s).

*(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e), can be filed subsequently).*

☐ Showing that the filing is authorized.  
*(not required unless called into question. 37 C.F.R. § 1.41(d))*

## 6. Inventorship Statement

**WARNING:** *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

☐ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

☐ is submitted.

☐ will be submitted.

## 7. Language

**NOTE:** *An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of \$130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).*

☒ English

☐ Non-English

☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

☒ An assignment of the invention to Children's Medical Center Corporation

☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

☒ will follow.

**NOTE:** "If an assignment is submitted with a new application, send two separate letters—one for the application and one for the assignment" Notice of May 4, 1990 (1114 O.G. 77-78).

**WARNING:** A newly executed "STATEMENT UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.

9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. no.	Filed
Country	Appln. no.	Filed
Country	Appln. no.	Filed

from which priority is claimed

☐ is (are) attached.

☐ will follow.

**NOTE:** The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

**NOTE:** This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. ☒ Regular application

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CLAIMS AS FILED

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Claims	Number Filed	Basic Fee Allowance	Number Extra	Rate	Basic Fee 37 C.F.R. § 1.16(a) \$760.00
<hr/>					
Total Claims					
(37 C.F.R. § 1.16(c))		- 20 =	x	\$ 18.00	
<hr/>					
Independent Claims					
(37 C.F.R. § 1.16(b))		- 3 =	x	\$ 78.00	
<hr/>					
Multiple Dependent Claim(s), if any			+	\$260.00	
(37 C.F.R. § 1.16(d))					

000050" E0808560

- | Variable                         | Mean | SD   | Min | Max |
|----------------------------------|------|------|-----|-----|
| Age                              | 34.2 | 10.5 | 18  | 65  |
| Gender                           | 0.5  | 0.5  | 0   | 1   |
| Marital status                   | 0.6  | 0.5  | 0   | 1   |
| Education                        | 12.5 | 1.5  | 9   | 16  |
| Income                           | 15.2 | 5.8  | 5   | 35  |
| Health status                    | 1.2  | 0.8  | 0   | 3   |
| Stress level                     | 2.1  | 1.2  | 0   | 4   |
| Life satisfaction                | 3.5  | 1.0  | 1   | 5   |
| Work engagement                  | 2.8  | 1.1  | 0   | 4   |
| Organizational commitment        | 3.2  | 1.2  | 1   | 5   |
| Turnover intention               | 1.5  | 0.9  | 0   | 3   |
| Job satisfaction                 | 3.8  | 1.1  | 1   | 5   |
| Perceived organizational support | 3.1  | 1.3  | 1   | 5   |
| Psychological contract breach    | 1.8  | 1.0  | 0   | 3   |
| Trust in supervisor              | 3.4  | 1.2  | 1   | 5   |
| Organizational justice           | 3.6  | 1.1  | 1   | 5   |
| Employee voice                   | 2.9  | 1.0  | 0   | 4   |
| Employee silence                 | 1.7  | 0.9  | 0   | 3   |
| Employee withdrawal              | 1.4  | 0.8  | 0   | 3   |
| Employee citizenship             | 2.6  | 1.0  | 0   | 4   |
| Employee turnover                | 0.3  | 0.5  | 0   | 1   |

### 11. Small Entity Statement(s)

(Application Transmittal—page 8 of 12)

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100
1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	

Filing Fee Calculation (50% of **A**, **B** or **C** above) \$\_\_\_\_\_

## 12. Request for International-Type Search (37 C.F.R. § 1.104(d))

**13. Fee Payment Being Made at This Time**

[ ] Fee for international-type search report  
(\$40.00; 37 C.F.R. § 1.21(e)) \$ \_\_\_\_\_

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. § 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total Fees Enclosed \$ \_\_\_\_\_

#### 14. Method of Payment of Fees

- [ ] Check in the amount of \$ \_\_\_\_\_.
- [ ] Charge Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_.  
A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

#### 15. Authorization to Charge Additional Fees

**WARNING:** If no fees are to be paid on filing, the following items should not be completed.

**WARNING:** Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.

- [ ] The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. \_\_\_\_\_.  
[ ] 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)  
[ ] 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

- [ ] 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)  
[ ] 37 C.F.R. § 1.17(a)(1)-(5) (extension fees pursuant to § 1.136(a)).  
[ ] 37 C.F.R. § 1.17 (application processing fees)

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for

*extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission.” 37 C.F.R. § 1.136(a)(3).*

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

*NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b)).*

*NOTE: 37 C.F.R. § 1.28(b) requires “Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . issue fee.” From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as “other than a small entity” and (b) no notification is required if the change is to another small entity.*

## **16. Instructions as to Overpayment**

*NOTE: “. . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account.” 37 C.F.R. § 1.26(a).*

☐ Credit Account No. \_\_\_\_\_.

☐ Refund

  
SIGNATURE OF PRACTITIONER

Reg. No. 34,235  
Tel. No.: (617) 345-6073

David S. Resnick  
NIXON PEABODY LLP  
101 Federal Street  
Boston, MA 02110

Customer No.:

**[X] Incorporation by reference of added pages**

*(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)*

[X ] Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

[ ] Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added \_\_\_\_\_

[ ] Plus added pages deleting names of inventor(s) named on prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added \_\_\_\_\_

[ ] Plus “Assignment Cover Letter Accompanying New Application”

Number of pages added \_\_\_\_\_

**[ ] Statement Where No Further Pages Added**

*(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)*

[ ] This transmittal ends with this page.

*NOTE: See 37 C.F.R. § 1.78.*

**WARNING:** *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

[X] Amend the specification by inserting, before the first line, the following sentence:

NOTE: “Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number).” 37 C.F.R. § 1.78(a)(4).

[ ] "This application claims the benefit of U.S. Provisional Application(s) No(s):

**FILING DATE**

\_\_\_\_\_ / \_\_\_\_\_

\_\_\_\_\_ / \_\_\_\_\_

\_\_\_\_\_ / \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

[X] "This application is a

[X ]continuation

[illegible]

☐ continuation-in-part

☐ divisional

of copending application(s)

☐ application number 0 / \_\_\_\_\_ filed on \_\_\_\_\_ ”

☒ International Application PCT/US98/ 26114 filed on 9 December 1998  
and which designated the U.S.”

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

“The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application.”

☒ “The nonprovisional application designated above, namely application  
PCT / US98/26114, filed 9 December 1998,  
claims the benefit of U.S. Provisional Application(s) No(s).:

**APPLICATION NO(S).:**

**FILING DATE**

<u>60 / 069,155</u>	<u>9 December 1997</u> ”
<u>60 / 069,687</u>	<u>12 December 1997</u> ”
<u>60 / 078,541</u>	<u>19 March 1998</u> ”

☒ Where more than one reference is made above please combine all references into one sentence.

## 18. Relate Back—35 U.S.C. 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed
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The certified copy(ies) has (have)

☐ been filed on \_\_\_\_\_, in prior application 0 / \_\_\_\_\_, which was filed on \_\_\_\_\_.

☐ is (are) attached.

**WARNING:** *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may not be relied on without any need to file a certified copy of the priority application in the continuing application. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

## 19. Maintenance of Copendency of Prior Application

**NOTE:** *The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).*

A. ☐ Extension of time in prior application

*(This item must be completed and the papers filed in the prior application, if the period set in the prior application has run.)*

☐ A petition, fee and response extends the term in the pending **prior** application until \_\_\_\_\_

☐ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

*(complete this item, if previous item not applicable)*

☐ A conditional petition for extension of time is being filed in the pending **prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

**20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed**

*(complete applicable item (a), (b) and/or (c) below)*

- (a) ☐ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

☐ the same.

- ☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

---

*(type name(s) of inventor(s) to be deleted)*

- (b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

☐ the same.

- ☐ the following additional inventor(s) have been added:

---

*(type name(s) of inventor(s) to be deleted)*

- (c) ☐ The inventorship for all the claims in this application are

☐ the same.

- ☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made

☐ is submitted.

☐ will be submitted.

**21. Abandonment of Prior Application *(if applicable)***

- ☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

**NOTE:** According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

## 22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

**WARNING:** *"The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." MPEP, § 706.07(b), 6th ed., rev.2.*

**NOTE:** *Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.*

*(check the next item, if applicable)*

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

## 23. Small Entity (37 CFR § 1.28(a))

☐ Applicant has established small entity status by the filing of a statement in parent application  
/ \_\_\_\_\_ on \_\_\_\_\_.

☐ A copy of the statement previously filed is included.

**WARNING:** *See 37 CFR § 1.28(a).*

## 24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this  
*(check one of the following)*

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Michael KLAGSBRUN, Shay SOKER, Hua-Quan MIAO, and Seiji TAKASHIMA  
Application No.:  
Filed: Herewith  
For: ANTAGONISTS OF NEUROPILIN RECEPTOR FUNCTION AND USE THEREOF



Assistant Commissioner for Patents  
Washington, D.C. 20231

EXPRESS MAIL CERTIFICATE

"Express Mail" label number EK571074376US  
Date of Deposit 05/30/2000

I hereby state that the following *attached* paper or fee

1. New Application Transmittal (12 pages)
2. Added Pages for Application Transmittal where Benefit of Prior US Applications Claimed (5 pages)
3. Specification (46 pages)
4. Claims (2 pages)
5. Drawings (21 pages)
6. Abstract (1 page)
7. Sequence Listing (9 pages)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Nicholas A. Zachariades

A handwritten signature in black ink, appearing to read "N. Zachariades", written over a horizontal line.

Signature of person mailing paper or fee

ANTAGONISTS OF NEUROPILIN RECEPTOR FUNCTION AND USE THEREOF

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The work described herein was supported, in part, by National Institute of Health grants CA37392 and CA45548. The U.S. Government has certain rights to the invention.

## 5 FIELD OF THE INVENTION

The present invention relates to antagonists of neuropilin receptor function and use thereof in the treatment of cancer, particularly metastatic cancer, and angiogenic diseases.

## 10 BACKGROUND OF THE INVENTION

Cancer, its development and treatment is a major health concern. The standard treatments available are few and directed to specific types of cancer, and provide no absolute guarantee of success. Most treatments rely on an approach that involves killing off rapidly growing cells in the hope that rapidly growing cancerous cells will succumb, either to the treatment, or at least be sufficiently reduced in numbers to allow the body's system to eliminate the remainder. However most, of these treatments are non-specific to cancer cells and adversely effect non-malignant cells. Many cancers although having some phenotype relationship are quite diverse. Yet, what treatment works most effectively for one cancer may not be the best means for treating another cancer. Consequently, an appreciation of the severity of the condition must be made before beginning many therapies. In order to most effective, these treatments require not only an early detection of the malignancy, but an appreciation of the severity of the malignancy. Currently, it can be difficult to distinguish cells at a molecular level as it relates to effect on treatment. Thus, methods of being able to screen malignant cells and better understand their disease state are desirable.

While different forms of cancer have different properties, one factor which many cancers share is that they can metastasize. Until such time as metastasis occurs, a tumor, although it may be malignant, is confined to one area of the body. This may cause discomfort and/or pain, or even lead to more serious problems including death, but if it can be located, it may be surgically removed and, if done with adequate care, be treatable. However, once metastasis sets in, cancerous cells have invaded the body and while surgical resection may remove the parent tumor, this does not address other tumors. Only chemotherapy, or some particular form of targeting therapy, then stands any chance of success.

- 10       The process of tumor metastasis is a multistage event involving local invasion and destruction of intercellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler, et al., *Adv. Cancer Res.* 28, 149-250 (1978), Liotta, et al., *Cancer Treatment Res.* 40, 223-238 (1988),  
15   Nicolson, *Biochim. Biophys. Acta* 948, 175-224 (1988) and Zetter, *N. Eng. J. Med.* 322, 605-612 (1990)). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially. Common to many steps of the metastatic process is a requirement for motility. The enhanced movement of malignant tumor cells is a major contributor to the progression of the disease toward metastasis.  
20   Increased cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka, et al., *Gann* 69, 273-276 (1978) and Haemmerlin, et al., *Int. J. Cancer* 27, 603-610 (1981)).

- Identifying factors that are associated with onset of tumor metastasis is extremely important. In addition, to using such factors for diagnosis and prognosis, those factors are an important site for identifying new compounds that can be used for treatment and as a target for treatment identifying new modes of treatment such as inhibition of metastasis is highly desirable.

- Tumor angiogenesis is essential for both primary tumor expansion and metastatic tumor spread, and angiogenesis itself requires ECM degradation (Blood et al., *Biochim. Biophys. Acta* 1032:89-118 (1990)). Thus, malignancy is a systemic disease in which interactions between the neoplastic cells and their environment play a



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encoded domain which is absent in VEGF<sub>121</sub> (Soker, et al., *J. Biol. Chem.*, 271:5761-5767 (1996)). However, the function of the receptor was unclear.

Identifying the alterations in gene expression which are associated with malignant tumors, including those involved in tumor progression and angiogenesis, is clearly a prerequisite not only for a full understanding of cancer, but also to develop new rational therapies against cancer.

A further problem arises, in that the genes characteristic of cancerous cells are very often host genes being abnormally expressed. It is quite often the case that a particular protein marker for a given cancer while expressed in high levels in connection with that cancer is also expressed elsewhere throughout the body, albeit at reduced levels.

The current treatment of angiogenic diseases is inadequate. Agents which prevent continued angiogenesis, e.g, drugs (TNP-470), monoclonal antibodies, antisense nucleic acids and proteins (angiostatin and endostatin) are currently being tested. See, Battegay, *J. Mol. Med.*, 73, 333-346 (1995); Hanahan et al., *Cell*, 86, 353-364 (1996); Folkman, *N. Engl. J. Med.*, 333, 1757-1763 (1995). Although preliminary results with the antiangiogenic proteins are promising, there is still a need for identifying genes encoding ligands and receptors involved in angiogenesis for the development of new antiangiogenic therapies.

## SUMMARY OF THE INVENTION

We have isolated a cDNA encoding the VEGF<sub>165</sub> R gene (SEQ ID NO: 1) and have deduced the amino acid sequence of the receptor (SEQ ID NO:2) We have discovered that this novel VEGF receptor is structurally unrelated to Flt-1 or KDR/Flk-1 and is expressed not only by endothelial cells but by non-endothelial cells, including surprisingly tumor cells.

In ascertaining the function of the VEGF<sub>165</sub>R we have further discovered that this receptor has been identified as a cell surface mediator of neuronal cell guidance and called neuropilin-1. Kolodkin et al., *Cell* 90:753-762 (1997). We refer to the receptor as VEGF<sub>165</sub>R/NP-1 or NP-1.

In addition to the expression cloning of VEGF<sub>165</sub>R/NP-1 cDNA we isolated another human cDNA clone whose predicted amino acid sequence was 47% homologous to that of VEGF<sub>165</sub>R/NP-1 and over 90% homologous to rat neuropilin-2 (NP-2) which was recently cloned (Kolodkin, et al., *Cell* 90, 753-762 (1997)).

5 Our results indicate that VEGF<sub>165</sub>R/NP-1 and NP-2 are expressed by both endothelial and tumor cells. (Fig. 19) We have shown that endothelial cells expressing both KDR and VEGF<sub>165</sub>R/NP-1 respond with increased chemotaxis towards VEGF<sub>165</sub>, not VEGF<sub>121</sub>, when compared to endothelial cells expressing KDR alone. While not wishing to be bound by theory, we believe that VEGF<sub>165</sub>R/NP-1 functions in  
10 endothelial cells to mediate cell motility as a co-receptor for KDR.

We have also shown in the Boyden chamber motility assay that VEGF<sub>165</sub> stimulates 231 breast carcinoma cell motility in a dose-response manner (Fig 15A). VEGF<sub>121</sub> had no effect motility of these cells (Fig 15B). Since tumor cells such as, 231 cells, do not express the VEGF receptors, KDR or Flt-1, while not wishing to be  
15 bound by theory, we believe that tumor cells are directly responsive to VEGF<sub>165</sub> via VEGF<sub>165</sub>R/NP-1.

We have also analyzed two variants of Dunning rat prostate carcinoma cells, AT2.1 cells, which are of low motility and low metastatic potential, and AT3.1 cells, which are highly motile, and metastatic. Cross-linking and Northern blot analysis  
20 show that AT3.1 cells express abundant VEGF<sub>165</sub>R/NP-1, capable of binding VEGF<sub>165</sub>, while AT2.1 cells don't express VEGF<sub>165</sub>R/NP-1 (Fig 18). Immunostaining of tumor sections confirmed the expression of VEGF<sub>165</sub>R/NP-1 in AT3.1, but not AT2.1 tumors (Fig 17). Additionally, immunostaining showed that in subcutaneous AT3.1 and PC3 tumors, the tumor cells expressing VEGF<sub>165</sub>R/NP-1 were found  
25 preferentially at the invading front of the tumor/dermis boundary (Fig 17). Furthermore, stable clones of AT2.1 cells overexpressing VEGF<sub>165</sub>R/NP-1 had enhanced motility in the Boyden chamber assay. These results indicate that neuropilin expression on tumor cells is associated with the motile, metastatic phenotype and angiogenesis, and thus is an important target for antiangiogenic and anticancer  
30 therapy.

The present invention relates to antagonists of neuropilin (NP) receptor function that can be use to inhibit metastasis and angiogenesis. Antagonists of invention can block the receptor preventing ligand binding, disrupt receptor function, or inhibit receptor occurrence. Specific antagonists include, for example, compounds that bind to NP-1 or NP-2 and antibodies that specifically binds the receptor at a region that inhibits receptor function. For example, one can add an effective amount of a compound that binds to NP-1 to disrupt receptor fuction and thus inhibit metastasis.

We have surprisingly discovered that members of the semaphorin/collapsins family are not only inhibitors of neuronal guidance but also inhibitors of endothelial and tumor cell motility in cells that express neuropilin. Accordingly, preferred antagonists include members of the semaphorin/collapsins family or fragments thereof that bind NP and have VEGF antagonist activity as determined, for example, by the human umbilical vein endothelial cell (HUVEC) proliferation assay using VEGF<sub>165</sub> as set forth in Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997). Preferably, the semaphorin/collapsin has at least a 25% reduction in HUVEC proliferation, more preferably a 50% reduction, even more preferably a 75% reduction, most preferably a 95% reduction.

VEGF antagonist activity of the semaphorin/collapsin may also be determined by inhibition of binding of labeled VEGF<sub>165</sub> to VEGF<sub>165</sub>R as disclosed in Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)) or to PAE/NP cells. Preferably, the portion inhibits binding by at least 25%, more preferably 50%, most preferably 75%.

In accordance with the present invention, neuropilin antagonists, or nucleic acids, e.g., DNA or RNA, encoding such antagonists, are useful as inhibitors of VEGF and NP function and can be used to treat diseases, disorders or conditions associated with VEGF and NP expression. The antagonists can be used alone or in combination with other anti-VEGF strategies including, for example, those that antagonize VEGF directly (e.g. anti-VEGF antibodies, soluble VEGF receptor extracellular domains), or antagonize VEGF receptors (e.g. anti-KDR antibodies, KDR kinase inhibitors, dominant-negative VEGF receptors) (Presta LG, et al., *Cancer Res.* 57: 4593-4599 (1997), Kendall RL, et al., (1996) *Biochem. Biophys. Res. Commun.* 226: 324-328,

Goldman CK, et al., (1998) Proc. Natl. Acad. Sci. USA 95: 8795-8800, Strawn LM, et al., (1996) Cancer Res. 56: 3540-3545, Zhu Z, et al., (1998). Cancer Res. 58: 3209-3214, Witte L, et al., (1998). Cancer Metastasis Rev. 17: 155-161.)

5 Diseases, disorders, or conditions, associated with VEGF, include, but are not limited to retinal neovascularization, hemangiomas, solid tumor growth, leukemia, metastasis, psoriasis, neovascular glaucoma, diabetic retinopathy, rheumatoid arthritis, endometriosis, mucular degeneration, osteoarthritis, and retinopathy of prematurity (ROP).

10 In another embodiment, one can use isolated VEGF<sub>165</sub>R/NP-1 or NP-2 or cells expressing these receptors in assays to discover compounds that bind to or otherwise interact with these receptors in order to discover NP antagonists that can be used to inhibit metastasis and/or angiogenesis.

Other aspects of the invention are disclosed *infra*.

## 15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows purification of VEGF<sub>165</sub>R From 231 Cells.  
20 <sup>125</sup>I-VEGF<sub>165</sub> (5 ng/ml) was bound and cross-linked to receptors on 231 cells and analyzed by SDS PAGE and autoradiography (lane 1). VEGF<sub>165</sub>R was purified by Con A and VEGF<sub>165</sub> affinity column chromatography and analyzed by SDS-PAGE and silver stain (lane 2). Two prominent bands were detected (arrows) and N-terminally sequenced separately. Their N-terminal 18 amino acid sequences are shown to the right of the arrows. The published N-terminal sequences of human and mouse neuropilin (Kawakami et al., *J. Neurobiol.*, 29, 1-17 (1995); He and Tessier-Lavigne, *Cell* 90, 739-751 1997) are shown below (SEQ ID NOS: 5 and 6).

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Figures 2A and 2B show isolation of VEGF<sub>165</sub>R cDNA by Expression Cloning. Photomicrographs (dark field illumination) of COS 7 cells binding <sup>125</sup>I-VEGF<sub>165</sub>. <sup>125</sup>I-VEGF<sub>165</sub> was bound to transfected COS 7 cells which were then washed, fixed, and overlaid with photographic emulsion that was developed as described in the  
30 example, *infra*.

Figure 2A shows COS 7 cells were transfected with a primary plasmid pool (#55 of the 231 cell library) representing approximately  $3 \times 10^3$  clones and one COS 7 cell binding  $^{125}\text{I}$ -VEGF<sub>165</sub> in the first round of screening is shown.

Figure 2 shows several COS 7 cells transfected with a single positive cDNA clone (A2) binding  $^{125}\text{I}$ -VEGF<sub>165</sub> after the third round of screening.

Figure 3 shows the Deduced Amino Acid Sequence of Human VEGF<sub>165</sub>R/NP-1 (SEQ ID NO:3). The deduced 923 amino acid sequence of the open reading frame of VEGF<sub>165</sub>R/NP-1, clone A2 (full insert size of 6.5 kb) is shown. The putative signal peptide sequence (amino acids 1-21) and the putative transmembrane region (amino acids 860-883) are in boxes. The amino acid sequence obtained by N-terminal amino acid sequencing (Figure 3, amino acids 22-39) is underlined. The arrow indicates where the signal peptide has been cleaved and removed, based on comparison of the N-terminal sequence of purified VEGF<sub>165</sub>R/NP-1 and the cDNA sequence. The sequence of human VEGF<sub>165</sub>R/NP-1 reported here differs from that reported by He et al. (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)) in that we find Lys<sub>26</sub> rather than Glu<sub>26</sub>, and Asp<sub>855</sub> rather than Glu<sub>855</sub>. Lys<sub>26</sub> and Asp<sub>855</sub> are found, however, in mouse and rat VEGF<sub>165</sub>R/NP-1 (Kwakami et al., *J. Neurobiol.* 29, 1-17 (1995); He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)).

Figure 4 shows the Comparison of the Deduced Amino Acid Sequence of Human VEGF<sub>165</sub>R/NP-1 (SEQ ID NO:2) and NP-2 (SEQ ID NO:4). The deduced open reading frame amino acid sequences of VEGF<sub>165</sub>R/NP-1 and NP-2 are aligned using the DNASIS program. Amino acids that are identical in both open reading frames are shaded. The overall homology between the two sequences is 43%.

Figure 5 shows a Northern Blot Analysis of VEGF<sub>165</sub>R/NP-1 Expression in Human EC and Tumor-Derived Cell Lines. Total RNA samples prepared from HUVEC (lane 1) and tumor-derived breast carcinoma, prostate carcinoma and melanoma cell lines as indicated (lanes 2-8) were resolved on a 1% agarose gel and blotted onto a GeneScreen nylon membrane. The membrane was probed with  $^{32}\text{P}$ -

labeled VEGF<sub>165</sub>R/NP-1 cDNA and exposed to X-ray film. Equal RNA loading was demonstrated by ethidium bromide staining of the gel prior to blotting. A major species of VEGF<sub>165</sub>R/NP-1 mRNA of approximately 7 kb was detected in several of the cell lines.

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Figure 6 shows a Northern Blot Analysis of VEGF<sub>165</sub>R/NP-1 and KDR mRNA in Adult Human Tissues. A pre-made Northern blot membrane containing multiple samples of human mRNA (Clontech) was probed with <sup>32</sup>P-labeled VEGF<sub>165</sub>R/NP-1 cDNA (top) as described in Fig 5, and then stripped and reprobed with <sup>32</sup>P-labeled  
10 KDR cDNA (bottom).

Figures 7A and 7B show a Scatchard Analysis of VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1. Figure 7A. Increasing amounts of <sup>125</sup>I-VEGF<sub>165</sub> (0.1-50 ng/ml) were added to subconfluent cultures of PAE cells transfected with human VEGF<sub>165</sub>R/NP-1  
15 cDNA (PAE/NP-1 cells) in 48 well dishes. Non-specific binding was determined by competition with a 200-fold excess of unlabeled VEGF<sub>165</sub>. After binding, the cells were washed, lysed and the cell-associated radioactivity was determined using a  $\gamma$  counter.

Figure 7B. The binding data shown in Figure 7A were analyzed by the method  
20 of Scatchard, and a best fit plot was obtained with the LIGAND program (Munson and Rodbard, 1980). PAE/NP-1 cells express approximately  $3 \times 10^5$  VEGF<sub>165</sub> binding sites per cell and bind <sup>125</sup>I-VEGF<sub>165</sub> with a  $K_d$  of  $3.2 \times 10^{-10}$  M.

Figure 8 shows cross-linking of VEGF<sub>165</sub> and VEGF<sub>121</sub> to PAE cells  
25 Expressing VEGF<sub>165</sub>R/NP-1 and/or KDR. <sup>125</sup>I-VEGF<sub>165</sub> (5 ng/ml) (lanes 1-6) or <sup>125</sup>I-VEGF<sub>121</sub> (10 ng/ml) (lanes 7-10) were bound to subconfluent cultures of HUVEC (lane 1), PC3 (lane 2), PAE (lanes 3 and 7), a clone of PAE cells transfected with human VEGF<sub>165</sub>R/NP-1 cDNA (PAE/NP-1) (lanes 4 and 8), a clone of PAE cells transfected with KDR (PAE/KDR) (lanes 5 and 9), and a clone of PAE/KDR cells  
30 transfected with human VEGF<sub>165</sub>R/NP-1 cDNA (PAE/KDR/NP-1) (lanes 6 and 10).

The binding was carried out in the presence of 1  $\mu\text{g/ml}$  heparin. At the end of a 2 hour incubation, each  $^{125}\text{I}$ -VEGF isoform was chemically cross-linked to the cell surface.

The cells were lysed and proteins were resolved by 6% SDS-PAGE. The polyacrylamide gel was dried and exposed to X-ray film. Solid arrows denote radiolabeled complexes containing  $^{125}\text{I}$ -VEGF and KDR, open arrows denote radiolabeled complexes containing  $^{125}\text{I}$ -VEGF and VEGF<sub>165</sub>R/NP-1.

Figure 9 shows cross linking of VEGF<sub>165</sub> to PAE/KDR Cells Co-expressing VEGF<sub>165</sub>R/NP-1 Transiently. PAE/KDR cells were transfected with pCPhygro or pCPhyg-NP-1 plasmids as described in "Experimental Procedures", and grown for 3 days in 6 cm dishes.  $^{125}\text{I}$ -VEGF<sub>165</sub> (5 ng/ml) was bound and cross linked to parental PAE/KDR cells (lane 1), to PAE/KDR cells transfected with pCPhygro vector control (V) (lane 2), to PAE/KDR cells transfected with pCPhyg- VEGF<sub>165</sub>R/NP-1 plasmids (VEGF<sub>165</sub>R/NP-1) (lane 3), and to HUVEC (lane 4). ). The binding was carried out in the presence of 1  $\mu\text{g/ml}$  heparin. The cells were lysed and proteins were resolved by 6% SDS-PAGE as in Figure 8. Solid arrows denote radiolabeled complexes containing  $^{125}\text{I}$ -VEGF<sub>165</sub> and KDR. Open arrows denote radiolabeled complexes containing  $^{125}\text{I}$ -VEGF<sub>165</sub> and VEGF<sub>165</sub>R/NP-1.

Figure 10 shows inhibition of  $^{125}\text{I}$ -VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1 interferes with its binding to KDR.  $^{125}\text{I}$ -VEGF<sub>165</sub> (5 ng/ml) was bound to subconfluent cultures of PAE transfected with human VEGF<sub>165</sub>R/NP-1 cDNA (PAE/NP-1) (lanes 1 and 2), PAE/KDR cells (lanes 3 and 4), and PAE/KDR cells transfected with human VEGF<sub>165</sub>R/NP-1 cDNA (PAE/KDR/NP-1) (lanes 5 and 16) in 35 mm dishes. The binding was carried out in the presence (lanes 2, 4, and 6) or the absence (lanes 1, 3, and 5) of 25  $\mu\text{g/ml}$  GST-Ex 7+8. Heparin (1  $\mu\text{g/ml}$ ) was added to each dish. At the end of a 2 hour incubation,  $^{125}\text{I}$ -VEGF<sub>165</sub> was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE as in Figure 9. Solid arrows denote radiolabeled complexes containing

$^{125}\text{I}$ -VEGF<sub>165</sub> and KDR, open arrows denote radiolabeled complexes containing  $^{125}\text{I}$ -VEGF<sub>165</sub> and VEGF<sub>165</sub>R/NP-1.

Figures 11A-C show a model for VEGF<sub>165</sub>R/NP-1 modulation of VEGF<sub>165</sub> Binding to KDR. 11A.Cells expressing KDR alone. 11B.Cells co-expressing KDR and VEGF<sub>165</sub>R/NP-1. 11C.Cells co-expressing KDR and VEGF<sub>165</sub>R/NP-1 in the presence of GST- Ex 7+8 fusion protein.

A single KDR receptor or a KDR-VEGF<sub>165</sub>R/NP-1 pair is shown in top portion. An expanded view showing several receptors is shown in the bottom portion. VEGF<sub>165</sub> binds to KDR via exon 4 and to VEGF<sub>165</sub>R/NP-1 via exon 7 (Keyt et al. *J. Biol. Chem.* 271,5638-5646 (1996b); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). A rectangular VEGF<sub>165</sub> molecule represents a suboptimal conformation that doesn't bind to KDR efficiently while a rounded VEGF<sub>165</sub> molecule represents one that fits better into a binding site. In cells expressing KDR alone, VEGF<sub>165</sub> binds to KDR in a sub-optimal manner. In cells co-expressing KDR and VEGF<sub>165</sub>R/NP-1, the binding efficiency of VEGF<sub>165</sub> to KDR is enhanced. It may be that the presence of VEGF<sub>165</sub>R/NP-1 increases the concentration of VEGF<sub>165</sub> on the cell surface, thereby presenting more growth factor to KDR. Alternatively, VEGF<sub>165</sub>R/NP-1 may induce a change in VEGF<sub>165</sub> conformation that allows better binding to KDR, or both might occur. In the presence of GST-Ex 7+8, VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1 is competitively inhibited and its binding to KDR reverts to a sub-optimal manner.

Figure 12 shows the human NP-2 amino acid sequence (SEQ ID NO:4).

Figures 13A and 13B show the human NP-2 DNA sequence (SEQ ID NO:3).

Figures 14A, 14B and 14C show the nucleotide (SEQ ID NO:1) and amino acid sequences (SEQ ID NO:2) of VEGF<sub>165</sub>R/NP-1.

5            Figures 16A, 16B and 16C show motility and neuropilin-1 expression of  
Dunning rat prostate carcinoma cell lines AT3-1 (high motility, high metastatic  
potential) and AT2.1 (low motility, low metastatic potential) cells. (Figure 16A)  
AT3.1 cells are more motile than AT2.1 cells in a Boyden chamber assay. 125I-  
VEGF<sub>165</sub> cross-links neuropilin-1 on AT3.1 cells but does not cross-link to AT2.1  
10        cells. (Figure 16C) AT3.1 but not AT2.1 cells express neuropilin-1, while both cell  
types express VEGF.

20            Figures 18A and 18B show overexpression of neuropilin-1 in AT2.1 cells. (Figure 18A) Western blot, (Figure 18B) motility activity. Three AT2.1 clones (lanes 4,5,6) express higher amounts of neuropilin-1 protein and are more motile compared to parental AT2.1 cells or AT2.1 vector (AT2.1/V) controls and approach AT3.1 cell neuropilin-1 levels and migration activity.

Human NP-1

Human NP-2

Reverse (1181-1162): 5' GTAGGTAGATGAGGCACTGA 3'. (SEQ ID NO:10)

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## DETAILED DESCRIPTION OF THE INVENTION

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Semaphorin/collapsins are known in the art and can be isolated from natural sources or produced using recombinant DNA methods. See, for example, U.S. Patent 5,807,826. Additionally, fragments of the semaphorin/collapsins may be used. For example, a 70 amino acid region within the semaphorin domain specifies the biological activities of three collapsin family members (Koppel, et al., *Neuron* 19: 531-537).

15 We have shown that when collapsin-1 was added to cultures of porcine  
endothelial cells (PAE) and PAE neuropilin-1 and/or KDR transfectants, <sup>125</sup>I-  
Collapsin was found to bind to PAE cells expressing neuropilin-1 but not to PAE cells  
expressing KDR. Furthermore, in a Boyden chamber assay, collapsin-1 inhibited the  
basal migration of PAE expressing neuropilin-1. by about 60-70%, but had no effect  
20 on parental PAE or PAE expressing KDR alone (Fig. 20). Inhibition was dose-  
dependent and half-maximal inhibition occurred with 50 collapsing units/ml (as  
measured on DRG, 1 CU = 3 ng/ml). Thus, semaphorin/collapsins inhibit the motility  
of non-neuronal cells as long as neuropilin-1 is expressed.

In accordance with yet another aspect of the present invention, there are  
30 provided isolated antibodies or antibody fragments which selectively binds the  
receptor. The antibody fragments include, for example, Fab, Fab', F(ab')<sub>2</sub> or Fv

Antibodies, or their equivalents, or other receptor antagonists may also be used in accordance with the present invention for the treatment or prophylaxis of cancers.

Prophylaxis may be appropriate even at very early stages of the disease, as it is not known what specific event actually triggers metastasis in any given case. Thus, administration of the antagonists which interfere with receptor activity, may be effected as soon as cancer is diagnosed, and treatment continued for as long as is necessary, preferably until the threat of the disease has been removed. Such treatment may also be used prophylactically in individuals at high risk for development of certain cancers, e.g., prostate or breast.

Chimeric and humanized antibodies are also within the scope of the invention. It is expected that chimeric and humanized antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody. A variety of approaches for making chimeric antibodies, comprising for example a non-human variable region and a human constant region, have been described. See, for example, Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 81,6851 (1985); Takeda, et al., Nature 314,452(1985), Cabilly et al., U.S. Pat. No. 4,816,567; Boss et al., U.S. Pat. No. 4,816,397; Tanaguchi et al., European Patent Publication EP 171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. Additionally, a chimeric antibody can be

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further "humanized" such that parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

10           The present invention further provides use of neuropilin for intracellular or extracellular targets to affect binding. Intracellular targeting can be accomplished through the use of intracellularly expressed antibodies referred to as intrabodies. Extracellular targeting can be accomplished through the use of receptor specific antibodies.

15           These methods can be used to inhibit metastasis in malignant cells as we have found that the presence of these receptors is positively correlated with metastasis. One can treat a range of afflictions or diseases associated with expression of the receptor by directly blocking the receptor. This can be accomplished by a range of different approaches. One preferred approach is the use of antibodies that specifically block  
20 VEGF binding to the receptor. For example, an antibody to the VEGF binding site. Antibodies to these receptors can be prepared by standard means. For example, one can use single chain antibodies to target these binding sites.

          The antibody can be administered by a number of methods. One preferred method is set forth by Marasco and Haseltine in PCT WO94/02610, which is  
25 incorporated herein by reference. This method discloses the intracellular delivery of a gene encoding the antibody. One would preferably use a gene encoding a single chain antibody. The antibody would preferably contain a nuclear localization sequence. One preferably uses an SV40 nuclear localization signal. By this method one can intracellularly express an antibody, which can block VEGF<sub>165</sub>R/NP-1 or NP-2  
30 functioning in desired cells.

Variable	Mean	SD	Min	Max
Age (years)	35.2	10.5	18	65
Gender (Male/Female)	15/15			
Education (years)	12.5	1.5	9	16
Occupation (Student/Worker)	10/5			
Marital status (Single/Married)	12/3			
Religion (Muslim/Hindu)	10/5			
Income (USD/month)	150	50	50	300
Health status (Healthy/Sick)	12/3			
Smoking status (Smoker/Non-smoker)	5/10			
Alcohol consumption (Yes/No)	3/12			
Exercise frequency (Times/week)	2.5	1.5	0	5
Dietary habits (Vegetarian/Non-vegetarian)	8/7			
Stress level (Low/High)	10/5			
Sleep quality (Good/Poor)	10/5			
Family size (Number of members)	3.5	1.5	1	6
Urban/Rural residence	10/5			
Transportation mode (Car/Bike/Walk)	5/10			
Health insurance (Yes/No)	10/5			
Medical history (Hypertension/Diabetes)	5/10			
Current medications (Yes/No)	3/12			
Physical activity level (Sedentary/Active)	10/5			
Mental health status (Stable/Unstable)	10/5			
Substance use (Cocaine/Heroin)	3/12			
Legal status (Criminal/Non-criminal)	5/10			
Employment status (Employed/Unemployed)	10/5			
Financial stability (Stable/Unstable)	10/5			
Social support (Strong/Weak)	10/5			
Life satisfaction (High/Low)	10/5			
Overall health score (1-10)	6.5	2.5	1	10

Variable	Mean	SD	Min	Max
Age (years)	35.2	10.5	18	65
Gender (Male/Female)	15/15			
Education (years)	12.5	1.5	9	16
Occupation (Student/Worker)	10/5			
Marital status (Single/Married)	12/3			
Religion (Muslim/Hindu)	10/5			
Income (USD/month)	150	50	50	300
Health status (Good/Bad)	12/3			
Smoking status (Smoker/Non-smoker)	5/10			
Alcohol consumption (Yes/No)	2/13			
Exercise frequency (Regular/Irregular)	8/7			
Dietary habits (Vegetarian/Non-vegetarian)	10/5			
Stress level (Low/High)	10/5			
Sleep quality (Good/Poor)	10/5			
Family size (1-3/4-6)	10/5			
Work hours (8-10/11-12)	10/5			
Travel frequency (Frequent/Rarely)	10/5			
Health insurance (Yes/No)	10/5			
Chronic diseases (Yes/No)	5/10			
Medication use (Yes/No)	5/10			
Physical activity (Sedentary/Active)	10/5			
Mental health (Stable/Unstable)	10/5			
Substance use (Alcohol/Drugs)	2/13			
Family support (Strong/Weak)	10/5			
Community involvement (Yes/No)	10/5			
Life satisfaction (High/Low)	10/5			
Overall health score (1-10)	6.5	2.5	1	10

Variable	Mean	SD	Min	Max
Age (years)	35.2	10.5	18	65
Gender (Male/Female)	15/15			
Education (years)	12.5	1.5	9	16
Occupation (Student/Worker)	10/5			
Marital status (Single/Married)	12/3			
Religion (Muslim/Hindu)	10/5			
Income (USD/month)	150	50	50	300
Health status (Healthy/Sick)	12/3			
Smoking status (Smoker/Non-smoker)	5/10			
Alcohol consumption (Yes/No)	3/12			
Exercise frequency (Times/week)	2.5	1.5	0	5
Dietary habits (Vegetarian/Non-vegetarian)	8/7			
Stress level (Low/High)	10/5			
Sleep quality (Good/Poor)	10/5			
Family size (Number of members)	3.5	1.5	1	6
Urban/Rural residence	10/5			
Transportation mode (Car/Bike/Walk)	5/10			
Health insurance (Yes/No)	10/5			
Medical history (Hypertension/Diabetes)	5/10			
Current medications (Yes/No)	3/12			
Physical activity level (Sedentary/Active)	10/5			
Mental health status (Stable/Unstable)	10/5			
Substance use (Cocaine/Heroin)	3/12			
Legal status (Criminal/Non-criminal)	5/10			
Employment status (Employed/Unemployed)	10/5			
Financial stability (Stable/Unstable)	10/5			
Social support (Strong/Weak)	10/5			
Life satisfaction (High/Low)	10/5			
Overall health score (1-10)	6.5	2.5	1	10

Variable	Mean	SD	Min	Max
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Marital status (Single/Married)	12/3			
Religion (Muslim/Hindu)	10/5			
Income (USD/month)	150	50	50	300
Health status (Good/Bad)	12/3			
Smoking status (Smoker/Non-smoker)	5/10			
Alcohol consumption (Yes/No)	2/13			
Exercise frequency (Regular/Irregular)	8/7			
Dietary habits (Vegetarian/Non-vegetarian)	10/5			
Stress level (Low/High)	10/5			
Sleep quality (Good/Poor)	10/5			
Family size (1-3/4-6)	10/5			
Parental education (High/Low)	10/5			
Parental occupation (Professional/Manual)	10/5			
Parental health status (Good/Bad)	10/5			
Parental smoking status (Smoker/Non-smoker)	5/10			
Parental alcohol consumption (Yes/No)	2/13			
Parental exercise frequency (Regular/Irregular)	8/7			
Parental dietary habits (Vegetarian/Non-vegetarian)	10/5			
Parental stress level (Low/High)	10/5			
Parental sleep quality (Good/Poor)	10/5			
Parental family size (1-3/4-6)	10/5			
Parental parental education (High/Low)	10/5			
Parental parental occupation (Professional/Manual)	10/5			
Parental parental health status (Good/Bad)	10/5			
Parental parental smoking status (Smoker/Non-smoker)	5/10			
Parental parental alcohol consumption (Yes/No)	2/13			
Parental parental exercise frequency (Regular/Irregular)	8/7			
Parental parental dietary habits (Vegetarian/Non-vegetarian)	10/5			
Parental parental stress level (Low/High)	10/5			
Parental parental sleep quality (Good/Poor)	10/5			
Parental parental family size (1-3/4-6)	10/5			
Parental parental parental education (High/Low)	10/5			
Parental parental parental occupation (Professional/Manual)	10/5			
Parental parental parental health status (Good/Bad)	10/5			
Parental parental parental smoking status (Smoker/Non-smoker)	5/10			
Parental parental parental alcohol consumption (Yes/No)	2/13			
Parental parental parental exercise frequency (Regular/Irregular)	8/7			
Parental parental parental dietary habits (Vegetarian/Non-vegetarian)	10/5			
Parental parental parental stress level (Low/High)	10/5			
Parental parental parental sleep quality (Good/Poor)	10/5			
Parental parental parental family size (1-3/4-6)	10/5			
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Parental parental parental parental sleep quality (Good/Poor)	10/5			
Parental parental parental parental family size (1-3/4-6)	10/5			
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Parental parental parental parental parental health status (Good/Bad)	10/5			
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Parental parental parental parental parental exercise frequency (Regular/Irregular)	8/7			
Parental parental parental parental parental dietary habits (Vegetarian/Non-vegetarian)	10/5			
Parental parental parental parental parental stress level (Low/High)	10/5			
Parental parental parental parental parental sleep quality (Good/Poor)	10/5			
Parental parental parental parental parental family size (1-3/4-6)	10/5			
Parental parental parental parental parental parental education (High/Low)	10/5			
Parental parental parental parental parental parental occupation (Professional/Manual)	10/5			
Parental parental parental parental parental parental health status (Good/Bad)	10/5			
Parental parental parental parental parental parental smoking status (Smoker/Non-smoker)	5/10			
Parental parental parental parental parental parental alcohol consumption (Yes/No)	2/13			
Parental parental parental parental parental parental exercise frequency (Regular/Irregular)	8/7			
Parental parental parental parental parental parental dietary habits (Vegetarian/Non-vegetarian)	10/5			
Parental parental parental parental parental parental stress level (Low/High)	10/5			
Parental parental parental parental parental parental sleep quality (Good/Poor)	10/5			
Parental parental parental parental parental parental family size (1-3/4-6)	10/5			
Parental parental parental parental parental parental parental education (High/Low)	10/5			
Parental parental parental parental parental parental parental occupation (Professional/Manual)	10/5			
Parental parental parental parental parental parental parental health status (Good/Bad)	10/5			
Parental parental parental parental parental parental parental smoking status (Smoker/Non-smoker)	5/10			
Parental parental parental parental parental parental parental alcohol consumption (Yes/No)	2/13			
Parental parental parental parental parental parental parental exercise frequency (Regular/Irregular)	8/7			
Parental parental parental parental parental parental parental dietary habits (Vegetarian/Non-vegetarian)	10/5			

Variable	Mean	SD	Min	Max
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Alcohol consumption (Yes/No)	2/13			
Exercise frequency (Regular/Irregular)	8/7			
Dietary habits (Vegetarian/Non-vegetarian)	10/5			
Stress level (Low/High)	10/5			
Sleep quality (Good/Poor)	10/5			
Family size (1-3/4-6)	10/5			
Work hours (8-10/11-12)	10/5			
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Health insurance (Yes/No)	10/5			
Chronic diseases (Yes/No)	5/10			
Medication use (Yes/No)	5/10			
Physical activity (Sedentary/Active)	10/5			
Mental health (Stable/Unstable)	10/5			
Substance use (Alcohol/Drugs)	2/13			
Family support (Strong/Weak)	10/5			
Community involvement (Yes/No)	10/5			
Life satisfaction (High/Low)	10/5			
Overall health score (1-10)	6.5	2.5	1	10



system may be used as desired, generally depending on what is required by the operator. Suitable systems may also be used to amplify the genetic material, but it is generally convenient to use *E. coli* for this purpose when only proliferation of the DNA is required.

5           The polypeptides and proteins may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or monoclonal antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel  
10 electrophoresis; and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

          The present invention also provides binding assays using VEGF<sub>165</sub>R/NP-1 or NP-2 that permit the ready screening for compounds which affect the binding of the  
15 receptor and its ligands, e.g., VEGF<sub>165</sub>. These assays can be used to identify compounds that modulate, preferably inhibit metastasis and/or angiogenesis. However, it is also important to know if a compound enhances metastasis so that its use can be avoided. For example, in a direct binding assay the compound of interest can be added before or after the addition of the labeled ligand, e.g., VEGF<sub>165</sub>, and the  
20 effect of the compound on binding or cell motility or angiogenesis can be determined by comparing the degree of binding in that situation against a base line standard with that ligand, not in the presence of the compound. The assay can be adapted depending upon precisely what is being tested.

          The preferred technique for identifying molecules which bind to the neuropilin  
25 receptor utilizes a receptor attached to a solid phase, such as the well of an assay plate. The binding of the candidate molecules, which are optionally labeled (e.g., radiolabeled), to the immobilized receptor can be measured. Alternatively, competition for binding of a known, labeled receptor ligand, such as I-<sup>125</sup> VEGF<sub>165</sub>, can be measured. For screening for antagonists, the receptor can be exposed to a  
30 receptor ligand, e.g., VEGF<sub>165</sub>, followed by the putative antagonist, or the ligand and antagonist can be added to the receptor simultaneously, and the ability of the

The ability of discovered antagonists to influence angiogenesis or metastasis can also be determined using a number of know *in vivo* and *in vitro* assays. Such assays are disclosed in Jain et al., *Nature Medicine* 3, 1203-1208(1997), and the examples.

Formulations may be any that are appropriate to the route of administration, and will be apparent to those skilled in the art. The formulations may contain a  
15 suitable carrier, such as saline, and may also comprise bulking agents, other medicinal preparations, adjuvants and any other suitable pharmaceutical ingredients. Catheters are one preferred mode of administration.

Neuropilin expression may also be inhibited *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. An antisense nucleic acid molecule which is complementary to a nucleic acid molecule encoding receptor can be designed based upon the isolated nucleic acid molecules encoding the receptor provided by the invention. An antisense nucleic acid molecule can comprise a nucleotide sequence which is complementary to a coding strand of a nucleic acid, e.g. complementary to an mRNA sequence, constructed according to the rules of Watson and Crick base pairing, and can hydrogen bond to the coding strand of the nucleic acid. The antisense sequence complementary to a sequence of an mRNA can be complementary to a sequence in the coding region of the mRNA or can be complementary to a 5' or 3' untranslated region of the mRNA. Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA,

5 (VEGF<sub>165</sub>R/NP-1) or SEQ ID NO:3 (NP-2). A nucleic acid is designed which has a sequence complementary to a sequence of the coding or untranslated region of the shown nucleic acid. Alternatively, an antisense nucleic acid can be designed based upon sequences of a VEGF<sub>165</sub>R gene, which can be identified by screening a genomic DNA library with an isolated nucleic acid of the invention. For example, the sequence  
10 of an important regulatory element can be determined by standard techniques and a sequence which is antisense to the regulatory element can be designed.

The antisense nucleic acids and oligonucleotides of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid or oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids e.g. phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acids and oligonucleotides can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e. nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). The antisense expression vector is introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics, Vol. 1 (1)1986.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides,

hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The antagonists of the invention are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of  
5 sustained release depots, intravenous injection, intranasal administration, and the like. Accordingly, antagonists of the invention may be administered as a pharmaceutical composition comprising the antibody or nucleic acid of the invention in combination with a pharmaceutically acceptable carrier. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and  
10 the like. Suitable carriers (excipients) include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for  
15 example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively one may incorporate or encapsulate the compounds such as an antagonist in a suitable  
20 polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet Registered TM minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care Registered TM (Allergan), Neodecadron Registered TM (Merck,  
25 Sharp & Dohme), Lacrilube Registered TM, and the like, or may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide an antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A  
30 thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

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The NP antagonists of the invention can be combined with a therapeutically effective amount of another molecule which negatively regulates angiogenesis which may be, but is not limited to, TNP-470, platelet factor 4, thrombospondin-1, tissue inhibitors of metalloproteases (TIMP1 and TIMP2), prolactin (16-Kd fragment),  
5 angiostatin (38-Kd fragment of plasminogen), endostatin, bFGF soluble receptor, transforming growth factor beta, interferon alfa, soluble KDR and FLT-1 receptors and placental proliferin-related protein.

An NP antagonist of the invention may also be combined with chemotherapeutic agents.

10 The DNA encoding an antagonist, e.g., a collapsin, can be used in the form of gene therapy and delivered to a host by any method known to those of skill in the art to treat disorders associated with VEGF.

The amount of an NP antagonist required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and  
15 condition of the subject, and other factors readily determined by one of ordinary skill in the art.

All references cited above or below are herein incorporated by reference.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not  
20 construed as a limitation thereof.

#### EXAMPLE 1

Experimental procedures

Materials

25

Cell culture media, lipofectin and lipofectamin reagents for transfection were purchased from Life Technologies. Human recombinant VEGF<sub>165</sub> and VEGF<sub>121</sub> were produced in Sf-21 insect cells infected with recombinant baculovirus vectors encoding either human VEGF<sub>165</sub> or VEGF<sub>121</sub> as previously described (Cohen et al., *Growth*  
30 *Factors*, 7, 131-138 (1992); Cohen et al., *J. Biol. Chem.*, 270, 11322-11326 (1995)). GST VEGF exons 7+8 fusion protein was prepared in E.Coli and purified as previously described (Soker et al., *J. Biol. Chem.*, 271, 5761-5767 (1996)). Heparin,

hygromycin B and protease inhibitors were purchased from Sigma (St. Louis, MO).  $^{125}\text{I}$ -Sodium,  $^{32}\text{P}$ -dCTP, and GeneScreen-Plus hybridization transfer membrane were purchased from DuPont NEN (Boston, MA). Disuccinimidyl suberate (DSS) and IODO-BEADS were purchased from Pierce Chemical Co. (Rockford, IL). Con A  
5 Sepharose was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). RNazol-B was purchased from TEL-TEST Inc. (Friendswood, TX). Silver Stain kit and Trans-Blot PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Multiple tissue northern blot membranes were purchased from Clontech (Palo Alto, CA). PolyA Tract mRNA isolation kits were purchased from  
10 Promega (Madison, WI). ReditPrime DNA labeling kits and molecular weight markers were purchased from Amersham (Arlington Heights, IL). Plasmids: pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA), and pCPhygro, containing the CMV promoter and encoding hygromycin B phosphorilase, was kindly provided by Dr. Urban Deutsch (Max Plank Institute, Bad Nauheim, Germany). Restriction  
15 endonucleases and Ligase were purchased from New England Biolabs, Inc (Beverly, MA). NT-B2 photographic emulsion and x-ray film were purchased from the Eastman Kodak company (Rochester NY).

#### Cell culture

20 Human umbilical vein EC (HUVEC) were obtained from American Type Culture Collection (ATCC) (Rockville, MD), and grown on gelatin coated dishes in M-199 medium containing 20% fetal calf serum (FCS) and a mixture of glutamine, penicillin and streptomycin (GPS). Basic FGF (2 ng/ml) was added to the culture medium every other day. Parental porcine aortic endothelial (PAE) cells and PAE cells  
25 expressing KDR (PAE/KDR) (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)) were kindly provided by Dr. Lena Claesson-Welsh and were grown in F12 medium containing 10% FCS and GPS. MDA-MB-231 cells and MDA-MB-453 cells were obtained from ATCC, and grown in DMEM containing 10% FCS and GPS. The human melanoma cell lines, RU-mel, EP-mel and WK-mel were kindly provided by  
30 Dr. Randolph Byer (Boston University Medical School, Boston, MA), and grown in DMEM containing 2% FCS, 8% calf serum and GPS. Human metastatic prostate

adenocarcinoma, LNCaP and prostate carcinoma, PC3 cells were kindly provided by Dr. Michael Freeman (Children's Hospital, Boston, MA), and grown in RPMI 1640 containing 5% FCS and GPS.

5 Purification and protein sequencing

Approximately  $5 \times 10^8$  MDA-MB-231 cells grown in 150 cm dishes were washed with PBS containing 5 mM EDTA, scraped and centrifuged for 5 min at 500g. The cell pellet was lysed with 150 ml of 20 mM HEPES, pH 8.0, 0.5% triton X-100 and protease inhibitors including 1 mM AEBSF, 5  $\mu$ g/ml leupeptin and 5  $\mu$ g/ml aprotinin for 30 min on ice, and the lysate was centrifuged at 30,000 x g for 30 min.  $MnCl_2$  and  $CaCl_2$  were added to the supernatant to obtain a final concentration of 1mM each. The lysate was absorbed onto a Con A Sepharose column (7 ml) and bound proteins were eluted with 15 ml 20 mM HEPES, pH 8.0, 0.2 M NaCl, 0.1% triton X-100 and 1 M methyl- $\alpha$ -D-mannopyranoside at 0.2 ml/min. The elution was repeated twice more at 30 minute intervals. The Con A column eluates were pooled and incubated for 12 h at 4°C with 0.5 ml of VEGF<sub>165</sub>- Sepharose beads, containing about 150  $\mu$ g VEGF<sub>165</sub>, prepared as described previously (Wilchek and Miron, *Biochem. Int.* 4, 629-635. (1982)). The VEGF<sub>165</sub>-Sepharose beads were washed with 50 ml of 20 mM HEPES, pH 8.0, 0.2 M NaCl and 0.1% triton X-100 and then with 25 ml of 20 mM HEPES, pH 8.0. The beads were boiled in SDS-PAGE buffer and bound proteins were separated by 6% SDS-PAGE. Proteins were transferred to a TransBlot PVDF membrane using a semi-dry electric blotter (Hoeffer Scientific), and the PVDF membrane was stained with 0.1% Coomassie Brilliant Blue in 40% methanol. The two prominent proteins in a 130-140 kDa doublet were cut out separately and N-terminally sequenced using an Applied Biosystems model 477A microsequenator as a service provided by Dr. William Lane of the Harvard Microchemistry facility (Cambridge, MA).

Expression cloning and DNA sequencing

Complementary DNA (cDNA) was synthesized from 5  $\mu$ g 231 mRNA. Double-stranded cDNA was ligated to *EcoRI* adaptors, and size-fractionated on a 5-20% potassium acetate gradient. DNA fragments larger than 2kb were ligated to an eukaryotic expression plasmid, pcDNA3.1. The plasmid library was transfected into

E.coli to yield a primary library of approximately  $1 \times 10^7$  individual clones. A portion of the transformed bacteria was divided into 240 pools, each representing approximately  $3 \times 10^3$  individual clones. DNA prepared from each pool was used to transfect COS-7 cells seeded in 12 well dishes, using the Lipofectin reagent according to the manufacturer's instructions. Three days after transfection, the cells were incubated on ice for 2 h with  $^{125}\text{I}$ -VEGF<sub>165</sub> (10 ng/ml) in the presence of 1  $\mu\text{g/ml}$  heparin, washed and fixed with 4% paraformaldehyde in PBS.  $^{125}\text{I}$ -VEGF<sub>165</sub> binding to individual cells was detected by overlaying the monolayers with photographic emulsion, NT-B2, and developing the emulsion after two days as described (Gearing et al.,1989). Seven positive DNA pools were identified and DNA from one of the positive pools was used to transform E.Coli . The E. coli were sub-divided into 50 separate pools and plated onto 50 LB ampicillin dishes, with each pool representing approximately 100 clones. DNA made from these pools was transfected into COS-7 cells which were screened for  $^{125}\text{I}$ -VEGF<sub>165</sub> binding as described above. Twenty positive pools were detected at this step, and their corresponding DNAs were used to transform *E. Coli*. Each pool was plated onto separate LB ampicillin dishes and DNA was prepared from 96 individual colonies and screened in a 96-well two dimensional grid for  $^{125}\text{I}$ -VEGF<sub>165</sub> binding to tranfected COS-7 cells as described above. Seven single clones were identified as being positive at this step. The seven positive plasmid clones were amplified and their DNA was analyzed by restriction enzyme digestion. Six clones showed an identical digestion pattern of digest and one was different. One clone from each group was submitted for automated DNA sequencing.

#### Northern Analysis

Total RNA was prepared from cells in culture using RNazol according to the manufacturer's instructions. Samples of 20  $\mu\text{g}$  RNA were separated on a 1% formaldehyde-agarose gel, and transferred to a GeneScreen-Plus membrane. The membrane was hybridized with a  $^{32}\text{P}$  labeled fragment of human VEGF<sub>165</sub>R/NP-1 cDNA, corresponding to nucleotides 63-454 in the ORF, at 63°C for 18 h. The membrane was washed and exposed to an x-ray film for 18 h. A commercially-obtained multiple human adult tissue mRNA blot (Clonotech, 2  $\mu\text{g/lane}$ ) was probed

for human NP-1 in a similar manner. The multiple tissue blot was stripped by boiling in the presence of 0.5% SDS and re-probed with a  $^{32}\text{P}$  labeled fragment of KDR cDNA corresponding to nucleotides 2841-3251 of the ORF (Terman et al., *Oncogene* 6, 1677-1683 (1991)).

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#### Transfection of PAE cells

Parental PAE cells and PAE cells expressing KDR (PAE/KDR) (Waltenberger et al., 1994) were obtained from Dr. Lena Claesson-Welsh. Human NP-1 cDNA was digested with *Xho*I and *Xba*I restriction enzymes and subcloned into the  
10 corresponding sites of pCPhygro, to yield pCPhyg-NP-1. PAE and PAE/KDR cells were grown in 6 cm dishes and transfected with 5  $\mu\text{g}$  of pCPhyg-NP-1 using Lipofectamine, according to the manufacturer's instructions. Cells were allowed to grow for an additional 48 h and the medium was replaced with fresh medium containing 200  $\mu\text{g}/\text{ml}$  hygromycin B. After 2 weeks, isolated colonies ( $5-10 \times 10^3$   
15 cell/colony) were transferred to separate wells of a 48 well dish and grown in the presence of 200  $\mu\text{g}/\text{ml}$  hygromycin B. Stable PAE cell clones expressing VEGF<sub>165</sub>R/NP-1 (PAE/NP-1) or co-expressing VEGF<sub>165</sub>R/NP-1 and KDR (PAE/KDR/NP-1) were screened for VEGF<sub>165</sub> receptor expression by binding and cross linking of  $^{125}\text{I}$ -VEGF<sub>165</sub>. For transient transfection, PAE/KDR cells were  
20 transfected with VEGF<sub>165</sub>R/NP-1 as described above and after three days  $^{125}\text{I}$ -VEGF<sub>165</sub> cross-linking analysis was carried out.

#### Radio-iodination of VEGF, binding and cross-linking experiments.

The radio-iodination of VEGF<sub>165</sub> and VEGF<sub>121</sub> using IODO-BEADS was  
25 carried out as previously described (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). The specific activity ranged from 40,000-100,000 cpm/ng protein. Binding and cross-linking experiments using  $^{125}\text{I}$ -VEGF<sub>165</sub> and  $^{125}\text{I}$ -VEGF<sub>121</sub> were performed as previously described (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). VEGF binding was quantitated by  
30 measuring the cell-associated radioactivity in a  $\gamma$ -counter (Beckman, Gamma 5500). The counts represent the average of three wells. All experiments were repeated at least

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### Purification of VEGF<sub>165</sub>R

Cross-linking of <sup>125</sup>I-VEGF<sub>165</sub> to cell surface receptors of 231 cells results in formation of a 165-175 kDa labeled complex (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). These cells have about 1-2 x 10<sup>5</sup> VEGF<sub>165</sub> binding sites/cell. In contrast to VEGF<sub>165</sub>, VEGF<sub>121</sub> does not bind to the 231 cells and does not form a ligand-receptor complex (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). The relatively high VEGF<sub>165</sub>R number and the lack of any detectable KDR or Flt-1 mRNA in 231 cells (not shown) suggested that these cells would be a useful source for VEGF<sub>165</sub>R purification. Preliminary characterization indicated that VEGF<sub>165</sub>R is a glycoprotein and accordingly, a 231 cell lysate prepared from approximately 5 x 10<sup>8</sup> cells was absorbed onto a Con A Sepharose column. Bound proteins, eluted from the Con A column, were incubated with VEGF<sub>165</sub>-Sepharose and the VEGF<sub>165</sub>- affinity purified proteins were analyzed by SDS-PAGE and silver staining (Figure 9, lane 2). A prominent doublet with a molecular mass of about 130-135 kDa was detected. This size is consistent with the formation of a 165-175 kDa complex of 40-45 kDa VEGF<sub>165</sub> bound to receptors approximately 130-135 kDa in size (Figure 9, lane 1). The two bands were excised separately and N-terminal amino acid sequencing was carried out (Figure 1, right). Both the upper and lower bands had similar N-terminal amino acid sequences which showed high degrees of sequence homology to the predicted amino acid sequences in the N-terminal regions of mouse (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995)) and human neuropilin-1 (NP-1) (He and Tessier-Lavigne, *Cell* 90739-751 (1997)).

### Expression cloning of VEGF<sub>165</sub>R from 231 cell-derived mRNA

Concomitant with the purification, VEGF<sub>165</sub>R was cloned by expression cloning (Aruffo and Seed, *Proc. Natl. Acad. Sci. USA* 84, 8573-8577 (1987a); Aruffo and Seed, *EMBO J.* 6, 3313-3316 (1987b); Gearing et al., *EMBO J.* 8, 3667-3676 (1989)). For expression cloning, 231 cell mRNA was used to prepare a cDNA library of approximately 10<sup>7</sup> clones in a eukaryotic expression plasmid. *E. coli* transformed with the plasmid library were divided into pools. The DNA prepared from each pool were transfected into COS-7 cells in separate wells and individual cells were screened

for the ability to bind  $^{125}\text{I}$ -VEGF<sub>165</sub> as detected by autoradiography of monolayers overlaid with photographic emulsion (Fig 2A). After three rounds of subpooling and screening, seven single positive cDNA clones were obtained. Figure 2B shows binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to COS-7 cells transfected with one of these single positive clones (clone A2).

Restriction enzyme analysis revealed that six of the seven positive single clones had identical restriction digestion patterns but that one clone had a pattern that was different (not shown). Sequencing of one of these similar cDNA clones, clone A2 (Figure 3), showed it to be identical to a sequence derived from a human-expressed sequence tag data bank (dbEST). This sequence also showed a high percentage of homology to the sequence of mouse neuropilin, NP-1 (Kawakami et al., *J. Neurobiol* 29, 1-17 (1995)). After we had cloned human VEGF<sub>165</sub>R, two groups reported the cloning of rat and human receptors for semaphorin III and identified them to be NP-1 (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). The 231 cell-derived VEGF<sub>165</sub>R cDNA sequence is virtually identical (see figure legend 3 for exceptions) to the human NP-1 sequence (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)). Significantly, the predicted amino acid sequence obtained by expression cloning (Figure 3) confirmed the identification of VEGF<sub>165</sub>R as NP-1 that was determined by N-terminal sequencing (Figure 1), and we have therefore named this VEGF receptor, VEGF<sub>165</sub>R/NP-1.

The human VEGF<sub>165</sub>R/NP-1 cDNA sequence predicts an open reading frame (ORF) of 923 amino acids with two hydrophobic regions representing putative signal peptide and transmembrane domains (Figure 3). Overall, the sequence predicts ectodomain, transmembrane and cytoplasmic domains consistent with the structure of a cell surface receptor. The N-terminal sequence obtained via protein purification as shown in Figure 1 is downstream of a 21 amino acid putative hydrophobic signal peptide domain, thereby indicating directly where the signal peptide domain is cleaved and removed. The short cytoplasmic tail of 40 amino acids is consistent with results demonstrating that soluble VEGF<sub>165</sub>R/NP-1 released by partial trypsin digestion of 231 cells is similar in size to intact VEGF<sub>165</sub>R/NP-1 (not shown).

Sequence analysis of the one clone obtained by expression cloning that had a different restriction enzyme profile predicted an open reading frame of 931 amino acids with about a 47% homology to VEGF<sub>165</sub>R/NP-1 (Figure 4). This human cDNA has a 93% sequence homology with rat neuropilin-2 (NP-2) and is identical to the recently cloned human NP-2 (Chen et al., *Neuron*, 19, 547-559 (1997)).

#### Expression of VEGF<sub>165</sub>R/NP-1 in adult cell lines and tissues

Reports of NP-1 gene expression have been limited so far to the nervous system of the developing embryo (Takagi et al., *Dev. Biol.* 122, 90-100 (1987); Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). Cell surface VEGF<sub>165</sub>R/NP-1, however, is associated with non-neuronal adult cell types such as EC and a variety of tumor-derived cells (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). Northern blot analysis was carried out to determine whether cells that crossed-linked <sup>125</sup>I-VEGF<sub>165</sub> also synthesized VEGF<sub>165</sub>R/NP-1 mRNA. (Figure 5). VEGF<sub>165</sub>R/NP-1 mRNA levels were highest in 231 and PC3 cells. VEGF<sub>165</sub>R/NP-1 mRNA was detected to a lesser degree in HUVEC, LNCaP, EP-mel and RU-mel cells. There was little if any expression in MDA-MB-453 and WK-mel cells. The VEGF<sub>165</sub>R/NP-1 gene expression patterns were consistent with our previous results showing that HUVEC, 231, PC3, LNCaP, EP-mel and RU-mel cells bind <sup>125</sup>I-VEGF<sub>165</sub> to cell surface VEGF<sub>165</sub>R/NP-1 but that MDA-MB-453 and WK-mel cells do not (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)).

VEGF<sub>165</sub>R/NP-1 gene expression was analyzed also by Northern blot in a variety of adult tissues in comparison to KDR gene expression (Figure 6). VEGF<sub>165</sub>R/NP-1 mRNA levels were relatively highly in adult heart and placenta and relatively moderate in lung, liver, skeletal muscle, kidney and pancreas. A relatively low level of VEGF<sub>165</sub>R/NP-1 mRNA was detected in adult brain. Interestingly, previous analysis of NP-1 gene expression in mouse and chicken brain suggested that this gene was expressed primarily during embryonic development and was greatly diminished after birth (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). The tissue distribution of KDR mRNA was similar to that of VEGF<sub>165</sub>R/NP-1 with the exception that it was not expressed highly in the

heart. These results indicate that VEGF<sub>165</sub>R/NP-1 is expressed widely in adult non-neuronal tissue, including tissues in which angiogenesis occurs such as heart and placenta.

5 Characterization of VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1

In order to characterize the binding properties of VEGF<sub>165</sub>R/NP-1, porcine aortic endothelial (PAE) cells were transfected with the cDNA of VEGF<sub>165</sub>R/NP-1. The PAE cells were chosen for these expression studies because they express neither KDR, Flt-1 (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)) nor

- 10 VEGF<sub>165</sub>R. Stable cell lines synthesizing VEGF<sub>165</sub>R/NP-1 (PAE/NP-1) were established and <sup>125</sup>I-VEGF<sub>165</sub> binding experiments were carried out (Fig 7). <sup>125</sup>I-VEGF<sub>165</sub> binding to PAE/NP-1 cells increased in a dose-dependent manner and reached saturation at approximately 30 ng/ml demonstrating that VEGF<sub>165</sub>R/NP-1 is a specific VEGF<sub>165</sub> receptor (Figure 7A). Scatchard analysis of VEGF<sub>165</sub> binding  
15 revealed a single class of VEGF<sub>165</sub> binding sites with a K<sub>d</sub> of approximately 3.2 x 10<sup>-10</sup> M and approximately 3 x 10<sup>5</sup> <sup>125</sup>I-VEGF<sub>165</sub> binding sites per cell (Figure 7B). Similar K<sub>d</sub> values were obtained for several independently-generated PAE/NP-1 clones, although the receptor number varied from clone to clone (not shown). The K<sub>d</sub> of 3 x 10<sup>-10</sup> M for the PAE/NP-1 cell lines is consistent with the 2-2.8 x 10<sup>-10</sup> M K<sub>d</sub>  
20 values obtained for VEGF<sub>165</sub>R/NP-1 expressed naturally by HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). The binding of <sup>125</sup>I-VEGF<sub>165</sub> to PAE/NP-1 cells was enhanced by 1 µg/ml heparin (not shown), consistent with previous studies showing that heparin enhances <sup>125</sup>I-VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1 on HUVEC and 231  
25 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)).

Isoform-specific binding of VEGF to cells expressing VEGF<sub>165</sub>R/NP-1

- VEGF<sub>165</sub>, but not VEGF<sub>121</sub>, binds to VEGF<sub>165</sub>R/NP-1 on HUVEC and 231  
30 cells (Gitay-Goren et al., *J. Biol. Chem.* 271, 5519-5523 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). To ascertain whether cells transfected with

VEGF<sub>165</sub>R/NP-1 had the same binding specificity, PAE/NP-1 cells were incubated with <sup>125</sup>I-VEGF<sub>165</sub> or <sup>125</sup>I-VEGF<sub>121</sub> followed by cross-linking (Figure 8). <sup>125</sup>I-VEGF<sub>165</sub> did not bind to parental PAE cells (Figure 8, lane 3) but did bind to PAE/NP-1 cells via VEGF<sub>165</sub>R/NP-1 (Figure 8, lane 4). The radiolabeled complexes formed with VEGF<sub>165</sub>R/NP-1 were similar in size to those formed in HUVEC (Figure 8, lane 1) and PC3 cells (Figure 8, lane 2). On the other hand, <sup>125</sup>I-VEGF<sub>121</sub>, did not bind to either parental PAE (Figure 8, lane 7) or to PAE/NP-1 cells (Figure 8, lane 8). These results demonstrate that the VEGF isoform-specific binding that occurs with cells expressing endogenous VEGF<sub>165</sub>R/NP-1 such as HUVEC, 231 and PC3 cells, can be replicated in cells transfected with VEGF<sub>165</sub>R/NP-1 cDNA and support the finding that VEGF<sub>165</sub>R and NP-1 are identical.

#### Co-expression of VEGF<sub>165</sub>R/NP-1 and KDR modulates VEGF<sub>165</sub> binding to KDR

To determine whether expression of VEGF<sub>165</sub>R/NP-1 had any effect on VEGF<sub>165</sub> interactions with KDR, PAE cells that were previously transfected with KDR cDNA to produce stable clones of PAE/KDR cells (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)), were transfected with VEGF<sub>165</sub>R/NP-1 cDNA and stable clones expressing both receptors (PAE/KDR/NP-1) were obtained. These cells bound <sup>125</sup>I-VEGF<sub>165</sub> to KDR (Figure 8, lane 6, upper complex) and to VEGF<sub>165</sub>R/NP-1 (Figure 8, lane 6, lower complex) to yield a cross-linking profile similar to HUVEC (Figure 8, lane 1). On the other hand, the PAE/KDR/NP-1 cells bound <sup>125</sup>I-VEGF<sub>121</sub> to form a complex only with KDR (Figure 8, lanes 9 and 10), consistent with the inability of VEGF<sub>121</sub> to bind VEGF<sub>165</sub>R/NP-1.

It appeared that in cells co-expressing KDR and VEGF<sub>165</sub>R/NP-1 (Figure 8, lane 6), the degree of <sup>125</sup>I-VEGF<sub>165</sub>-KDR 240 kDa complex formation was enhanced compared to the parental PAE/KDR cells (Figure 8, lane 5). These results were reproducible and the degree of <sup>125</sup>I-VEGF<sub>165</sub>-KDR 240 kDa complex formation in different clones was correlated positively with the levels of VEGF<sub>165</sub>R/NP-1 expressed (not shown). However, it could not be ruled out definitively that these differential KDR binding results were possibly due to clonal selection post-transfection.

Therefore, parental PAE/KDR cells were transfected with VEGF<sub>165</sub>R/NP-1 cDNA and <sup>125</sup>I-VEGF<sub>165</sub> was bound and cross-linked to the cells three days later in order to avoid any diversity of KDR expression among individual clones (Figure 9). A labeled 240 kDa complex containing KDR was formed in parental PAE/KDR cells (Fig 9, lane 1) and in PAE/KDR cells transfected with the expression vector (Figure 9, lane 2). However, when <sup>125</sup>I-VEGF<sub>165</sub> was cross-linked to PAE/KDR cells transiently expressing VEGF<sub>165</sub>R/NP-1, a more intensely labeled 240 kDa complex, about 4 times greater, was observed (Figure 9, lane 3), compared to parental PAE/KDR cells (Figure 9, lane 1) and PAE/KDR cells transfected with expression vector (Figure 9, lane 2). These results suggest that co-expression of KDR and VEGF<sub>165</sub>R/NP-1 genes in the same cell enhances the ability of VEGF<sub>165</sub> to bind to KDR.

#### A GST-VEGF Exon 7+8 fusion protein inhibits VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1 and KDR

We have shown that <sup>125</sup>I-VEGF<sub>165</sub> binds to VEGF<sub>165</sub>R/NP-1 through its exon 7-encoded domain (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). In addition, a GST fusion protein containing the peptide encoded by VEGF exon 7+8 (GST-Ex 7+8), inhibits completely the binding of <sup>125</sup>I-VEGF<sub>165</sub> to VEGF<sub>165</sub>R/NP-1 associated with 231 cells and HUVEC (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996); Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). When, added to PAE/NP-1 cells, the fusion protein completely inhibited binding to VEGF<sub>165</sub>R/NP-1 (Figure 10, lane 2 compared to lane 1). On the other hand, it did not inhibit <sup>125</sup>I-VEGF<sub>165</sub> binding at all to KDR (Figure 10, lane 4 compared to lane 3). Thus, these results demonstrate that GST-Ex 7+8 binds directly to VEGF<sub>165</sub>R/NP-1 but does not bind to KDR. The effects of GST-Ex 7+8 are different, however, in cells co-expressing both VEGF<sub>165</sub>R/NP-1 and KDR (PAE/KDR/NP-1). Consistent with the results in Figures 8 and 9, the degree of <sup>125</sup>I-VEGF<sub>165</sub> binding to KDR in PAE/KDR/NP-1 cells (Figure 10, lane 5) was greater than to the parental PAE/KDR cells (Figure 10, lane 3). Interestingly, in PAE/KDR/NP-1 cells, GST-Ex 7+8 inhibited not only <sup>125</sup>I-VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1 completely as expected, but it also inhibited binding to KDR substantially which was unexpected (Figure 10, lane 6 compared to lane 5). In the

Variable	Mean	SD	Min	Max
Age	38.5	10.2	25	55
Gender	0.5	0.5	0	1
Marital status	0.7	0.5	0	1
Education	12.5	1.5	10	15
Income	3500	1500	1000	6000
Health status	0.8	0.4	0	1
Exercise frequency	2.5	1.5	0	5
Stress level	4.5	1.5	1	7
Sleep quality	3.5	1.5	1	6
Work satisfaction	4.0	1.5	1	6
Life satisfaction	5.0	1.5	1	7

Table 1. (continued)	
Variable	Mean (SD)
Age (years)	34.5 (10.5)
Gender	
Male	100 (100%)
Female	0 (0%)
Marital status	
Married	100 (100%)
Single	0 (0%)
Education	
High school or less	100 (100%)
College or more	0 (0%)
Occupation	
Unemployed	100 (100%)
Employed	0 (0%)
Income (US\$)	
<1000	100 (100%)
1000-2000	0 (0%)
>2000	0 (0%)
Health status	
Good	100 (100%)
Poor	0 (0%)
Smoking status	
Smoker	100 (100%)
Nonsmoker	0 (0%)
Alcohol consumption	
Alcohol	100 (100%)
No alcohol	0 (0%)
Exercise	
Exercise	100 (100%)
No exercise	0 (0%)
Stress	
Stress	100 (100%)
No stress	0 (0%)
Depression	
Depression	100 (100%)
No depression	0 (0%)
Family size	
Family size	100 (100%)
No family size	0 (0%)
Family income	
Family income	100 (100%)
No family income	0 (0%)
Family structure	
Family structure	100 (100%)
No family structure	0 (0%)
Family health	
Family health	100 (100%)
No family health	0 (0%)
Family education	
Family education	100 (100%)
No family education	0 (0%)
Family occupation	
Family occupation	100 (100%)
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Family income	
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No family occupation	0 (0%)
Family income	
Family income	100 (100%)
No family income	0 (0%)
Family structure	
Family structure	100 (100%)
No family structure	0 (0%)
Family health	
Family health	100 (100%)
No family health	0 (0%)
Family education	

Variable	Mean	SD	Min	Max
Age	38.5	12.5	25	65
Gender	0.5	0.5	0	1
Marital status	0.5	0.5	0	1
Education	12.5	2.5	9	16
Income	1500	500	500	3000
Health status	0.5	0.5	0	1
Exercise frequency	0.5	0.5	0	1
Stress level	0.5	0.5	0	1
Sleep quality	0.5	0.5	0	1
Dietary habits	0.5	0.5	0	1
Alcohol consumption	0.5	0.5	0	1
Smoking status	0.5	0.5	0	1
Family size	2.5	1.5	1	5
Work hours	40	10	20	60
Commuting time	30	15	10	60
Childcare costs	500	200	0	1000
Health insurance	0.5	0.5	0	1
Access to green spaces	0.5	0.5	0	1
Proximity to public transport	0.5	0.5	0	1
Neighborhood safety	0.5	0.5	0	1
Availability of recreational facilities	0.5	0.5	0	1
Overall life satisfaction	0.5	0.5	0	1

Variable	Mean	SD	Min	Max
Age	38.5	10.2	25	55
Gender	0.5	0.5	0	1
Marital status	0.7	0.5	0	1
Education	12.5	1.5	10	15
Income	3500	1500	1000	6000
Health status	0.8	0.3	0	1
Exercise frequency	2.5	1.5	0	5
Stress level	4.5	1.5	1	7
Sleep quality	3.5	1.5	1	6
Diet quality	4.0	1.5	1	6
Alcohol consumption	1.5	1.5	0	4
Tobacco use	0.5	0.5	0	1
Family size	2.5	1.5	1	5
Work hours	40	5	30	50
Commuting time	30	10	10	60
Home ownership	0.8	0.4	0	1
Neighborhood safety	4.5	1.5	1	7
Access to green spaces	3.5	1.5	1	6
Public transportation	4.0	1.5	1	6
Crime rate	2.5	1.5	1	5
Healthcare access	4.5	1.5	1	7
Community engagement	3.5	1.5	1	6
Local government responsiveness	3.0	1.5	1	5
Environmental quality	4.0	1.5	1	6
Overall quality of life	4.5	1.5	1	7

(1996)). Cells that bound VEGF<sub>165</sub> to VEGF<sub>165</sub>R synthesized relatively abundant NP-1 mRNA while cells that showed very little if any VEGF<sub>165</sub> binding, did not synthesize much if any NP-1 mRNA; v) when NP-1 was expressed in PAE cells, the transfected, but not the parental cells, were able to bind VEGF<sub>165</sub> but not VEGF<sub>121</sub>, consistent with the isoform specificity of binding previously shown for HUVEC and 231 cells (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). Furthermore, the K<sub>d</sub> of <sup>125</sup>I-VEGF<sub>165</sub> binding of to PAE expressing NP-1 was about 3 x 10<sup>-10</sup> M, consistent with previous K<sub>d</sub> binding values of 2-2.8 x 10<sup>-10</sup> M for 231 cells and HUVEC (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)); and vi) The binding of VEGF<sub>165</sub> to cells expressing NP-1 post-transfection was more efficient in the presence of heparin as was the binding of this ligand to HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). Taken together, these results show not only that VEGF<sub>165</sub>R is identical to NP-1 but that it is a functional receptor that binds VEGF<sub>165</sub> in an isoform-specific manner. Accordingly, we have named this VEGF receptor VEGF<sub>165</sub>R/NP-1.

In addition to the expression cloning of VEGF<sub>165</sub>R/NP-1 cDNA, another human cDNA clone was isolated whose predicted amino acid sequence was 47% homologous to that of VEGF<sub>165</sub>R/NP-1 and over 90% homologous to rat neuropilin-2 (NP-2) which was recently cloned (Kolodkin et al., *Cell* 90, 753-762 (1997)). NP-2 binds members of the collapsin/semaphorin family selectively (Chen et al., *Neuron* 19, 547-559 (1997)).

The discovery that NP-1 serves as a receptor for VEGF<sub>165</sub> was a surprise since NP-1 had previously been shown to be associated solely with the nervous system during embryonic development (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)) and more recently as a receptor for members of the collapsin/semaphorin family (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). NP-1 is a 130-140 kDa transmembrane glycoprotein first identified in the developing *Xenopus* optic system (Takagi et al., *Dev. Biol.* 122, 90-100 (1987); Takagi et al., *Neuron* 7, 295-307 (1991)). NP-1 expression in the nervous system is highly regulated spatially and temporally during development and in particular is associated with those

5 *Neurobiol* 29, 1-17 (1995)). Functionally, neuropilin has been shown to promote neurite outgrowth of optic nerve fibers in vitro (Hirata et al., *Neurosci. Res.* 17, 159-169 (1993)) and to promote cell adhesiveness (Tagaki et al., *Dev. Biol.* 170, 207-222 (1995)). Targeted disruption of NP-1 results in severe abnormalities in the trajectory of efferent fibers of the peripheral nervous system (Kitsukawa et al., *Neuron* 19, 995-1005 (1997)). Based on the these studies, it has been suggested that NP-1 is a neuronal cell recognition molecule that plays a role in axon growth and guidance (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kitsukawa et al., *Neuron* 19, 995-1005 1997; Kolodkin et al., *Cell* 90, 753-762 (1997)).

30 In addition, NP-1 has been identified as a receptor for the  
collapsin/semaphorin family by expression cloning of a cDNA library obtained from

rat E14 spinal cord and dorsal root ganglion (DRG) tissue (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). The collapsin/semaphorins (collapsin-D-1/Sema III/Sem D) comprise a large family of transmembrane and secreted glycoproteins that function in repulsive growth cone and axon guidance (Kolodkin et al., *Cell* 75, 1389-1399 (1993)). The repulsive effect of sema III for DRG cells was blocked by anti-NP-1 antibodies (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). The  $K_d$  of sema III binding to NP-1,  $0.15-3.25 \times 10^{-10}$  M (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)) is similar to that of VEGF<sub>165</sub> binding VEGF<sub>165</sub>/NP-1, which is about  $3 \times 10^{-10}$  M. These results indicate that two structurally different ligands with markedly different biological activities, VEGF-induced stimulation of EC migration and proliferation on one hand, and sema III-induced chemorepulsion of neuronal cells, on the other hand, bind to the same receptor and with similar affinity. An interesting question is whether the two ligands bind to the same site on VEGF<sub>165</sub>R/NP-1 or to different sites. VEGF<sub>165</sub>R/NP-1 has five discrete domains in its ectodomain, and it has been suggested that this diversity of protein modules in NP-1 is consistent with the possibility of multiple binding ligands for NP-1 (Takagi et al., *Neuron* 7, 295-307 (1991); Feiner et al., *Neuron* 19 539-545 (1997); He and Tessier-Lavigne, *Cell* 90 739-751 (1997)). Preliminary analysis does not indicate any large degree of sequence homology between sema III and VEGF exon 7 which is responsible for VEGF binding to VEGF<sub>165</sub>R/NP-1 (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). However there may be some 3-dimensional structural similarities between the two ligands. Since both neurons and blood vessels display branching and directional migration, the question also arises as to whether VEGF<sub>165</sub> displays any neuronal guidance activity and whether sema III has any EC growth factor activity. These possibilities have not been examined yet. However, it may be that VEGF requires two receptors, KDR and NP-1 for optimal EC growth factor activity (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)) and that sema III requires NP-1 and an as yet undetermined high affinity receptor for optimal chemorepulsive activity (Feiner et al., *Neuron* 19, 539-545 (1997); He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kitsukawa et al., *Neuron* 19, 995-1005 (1997)), so

that the presence of NP-1 alone might not be sufficient for these ligands to display novel biological activities. Future studies will determine whether there are any connections between the mechanisms that regulate neurogenesis and angiogenesis.

5 VEGF<sub>165</sub>R/NP-1 role angiogenesis

VEGF<sub>165</sub>R/NP-1 modulates the binding of VEGF<sub>165</sub> to KDR, a high affinity RTK that is an important regulator of angiogenesis as evidenced by KDR knock out experiments in mice (Shalaby et al., *Nature* 376, 62-66 (1995)). The affinity of KDR for VEGF<sub>165</sub> is about 50 times greater than for VEGF<sub>165</sub>R/NP-1 (Gitay-Goren et al., *J. Biol. Chem.* 287, 6003-6096 (1992); Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)). When VEGF<sub>165</sub>R/NP-1 and KDR are co-expressed, the binding of <sup>125</sup>I-VEGF<sub>165</sub> to KDR is enhanced by about 4-fold compared to cells expressing KDR alone. The enhanced binding can be demonstrated in stable clones co-expressing VEGF<sub>165</sub>R/NP-1 and KDR (PAE/KDR/NP-1 cells), and also in PAE/KDR cells  
15 transfected transiently with VEGF<sub>165</sub>R/NP-1 cDNA where clonal selection does not take place. Conversely, when the binding of <sup>125</sup>I-VEGF<sub>165</sub> to VEGF<sub>165</sub>R/NP-1 in PAE/KDR/NP-1 cells is inhibited completely by a GST fusion protein containing VEGF exons 7+8 (GST-Ex 7+8), the binding to KDR is inhibited substantially, down to the levels observed in cells expressing KDR alone. The fusion protein binds to  
20 VEGF<sub>165</sub>R/NP-1 directly but is incapable of binding to KDR directly (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). Although, not wishing to be bound by theory, we believe that VEGF<sub>165</sub> binds to VEGF<sub>165</sub>R/NP-1 via the exon 7-encoded domain and facilitates VEGF<sub>165</sub> binding to KDR via the exon 4-encoded domain (Figure 11). VEGF<sub>165</sub>R/NP-1, with its relatively high receptor/cell number, about  $0.2-2 \times 10^5$   
25 (Gitay-Goren et al., *J. Biol. Chem.* 287, 6003-6096 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)), appears to serve to concentrate VEGF<sub>165</sub> on the cell surface, thereby providing greater access of VEGF<sub>165</sub> to KDR. Alternatively, binding to VEGF<sub>165</sub>R/NP-1, VEGF<sub>165</sub> undergoes a conformational change that enhances its binding to KDR. The end result would be elevated KDR signaling and increased  
30 VEGF activity. Although we can demonstrate enhanced binding to KDR, to date we have not been able to demonstrate enhanced VEGF mitogenicity for PAE/KDR/NP-1

cells compared to PAE/KDR cells. One reason is that these cell lines do not proliferate readily in response to VEGF as do HUVEC (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)). Nevertheless, we have shown that VEGF<sub>165</sub>, which binds to both KDR and VEGF<sub>165</sub>R/NP-1, is a better mitogen for HUVEC than is VEGF<sub>121</sub>, which binds only to KDR (Keyt et al., *J. Biol. Chem.* 271, 5638-5646 (1996b); Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). Furthermore, inhibiting VEGF<sub>165</sub> binding to VEGF<sub>165</sub>R/NP-1 on HUVEC by GST-EX 7+8, inhibits binding to KDR and also inhibits VEGF<sub>165</sub>-induced HUVEC proliferation, down to the level induced by VEGF<sub>121</sub> (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). Taken together, these results suggest a role for VEGF<sub>165</sub>R/NP-1 in mediating VEGF<sub>165</sub>, but not VEGF<sub>121</sub> mitogenic activity. The concept that dual receptors regulate growth factor binding and activity has been previously demonstrated for TGF- $\beta$ , bFGF and NGF (Lopez-Casillas et al., *Cell* 67, 785-795 (1991); Yaron et al., *Cell* 64, 841-848 (1991); Barbacid, *Curr. Opin. Cell Biol.* 7, 148-155 (1995)).

Another connection between VEGF<sub>165</sub>R/NP-1 and angiogenesis comes from studies in which NP-1 was overexpressed ectopically in transgenic mice (Kitsukawa et al., *Develop.* 121, 4309-4318 (1995)). NP-1 overexpression resulted in embryonic lethality and the mice died *in utero* no later than on embryonic day 15.5 and those that survived the best had lower levels of NP-1 expression. Mice overexpressing NP-1 displayed morphologic abnormalities in a limited number of non-neural tissues such as blood vessels, the heart and the limbs. NP-1 was expressed in both the EC and in the mesenchymal cells surrounding the EC. The embryos possessed excess and abnormal capillaries and blood vessels compared to normal counterparts and in some cases dilated blood vessels as well. Some of the chimeric mice showed hemorrhaging, mainly in the head and neck. These results are consistent with the possibility that ectopic overexpression of VEGF<sub>165</sub>R/NP-1 results in inappropriate VEGF<sub>165</sub> activity, thereby mediating enhanced and/or aberrant angiogenesis. Another piece of evidence for a link between NP-1 and angiogenesis comes from a recent report showing that in mice targeted for disruption of the NP-1 gene, the embryos have severe abnormalities in the peripheral nervous system but that their death *in utero* at days 10.5-12.5 is most

Variable	Mean	SD	Min	Max
Age	38.5	12.5	25	65
Gender	0.5	0.5	0	1
Marital status	0.6	0.5	0	1
Education	12.5	2.5	9	16
Income	3500	1500	1000	8000
Health status	0.8	0.2	0	1
Exercise frequency	2.5	1.5	0	5
Stress level	4.5	1.5	1	7
Sleep quality	3.5	1.5	1	6
Diet quality	4.0	1.5	1	6
Work-life balance	3.0	1.5	1	5
Overall well-being	4.5	1.5	1	7

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Work-life balance	3.0	1.5	1	5
Overall well-being	4.0	1.5	1	7

5 collapsins/semaphorins. Furthermore, binding to VEGF<sub>165</sub>R/NP-1 enhances the binding of VEGF<sub>165</sub> to KDR on EC and tumor cells.

We have discovered that tumor cell neuropilin-1 mediates tumor cell motility and thereby metastasis. In a Boyden chamber motility assay, VEGF<sub>165</sub> (50 ng/ml) stimulates 231 breast carcinoma cell motility in a dose-response manner, with a maximal 2-fold stimulation (Fig. 15A). On the other hand, VEGF<sub>121</sub> has no effect on motility of these cells (Fig. 15B). Since 231 cells do not express KDR or Flt-1, these results suggest that tumor cells are directly responsive to VEGF<sub>165</sub> and that VEGF<sub>165</sub> might signal tumor cells via neuropilin-1. Possible candidates for mediating VEGF<sub>165</sub>-induced motility of carcinoma cells are PI3-kinase (PI3-K) (Carpenter, et al. (1996) *Curr. Opin. Cell Biol.* 8: 153-158.). Since 231 cells do not express KDR or Flt-1, these results suggest that tumor cells are directly responsive to VEGF<sub>165</sub> and that VEGF<sub>165</sub> might signal tumor cells via neuropilin-1.

20 The other type of evidence is that neuropilin-1 expression might be associated with tumor cell motility. We have analyzed two variants of Dunning rat prostate carcinoma cells, AT2.1 cells, which are of low motility and low metastatic potential, and AT3.1 cells, which are highly motile, and metastatic. Cross-linking and Northern blot analysis show that AT3.1 cells express abundant neuropilin-1, capable of binding  
25 VEGF<sub>165</sub>, while AT2.1 cells don't express neuropilin-1 (Fig. 16). Immunostaining of tumor sections confirms the expression of neuropilin-1 in AT3.1, but not AT2.1 tumors (Fig. 17). Furthermore, the immunostaining shows that in subcutaneous AT3.1 and PC3 tumors, the tumor cells expressing neuropilin-1 are found preferentially at the invading front of the tumor/dermis boundary (Fig. 17). To determine more directly  
30 whether neuropilin-1 expression is correlated with enhanced motility, neuropilin-1 was overexpressed in AT2.1 cells (Fig. 18). Three stable clones of AT2.1 cells overexpressing neuropilin-1 had enhanced motility in the Boyden chamber assay.

## 5 EXAMPLE 2

1. Collapsin/semaphorins. Expression plasmids for expressing and purifying His-tagged collapsin-1 from transfected 293T cells can be produced according to the methods of (Koppel, et al. (1998) *J. Biol. Chem.* 273: 15708-15713, Feiner, et al. (1997) *Neuron* 19: 539-545.). Expression vectors for expressing sema E and sema IV alkaline phosphate (AP) conjugates in cells are disclosed in (He Z, Tessier-Lavigne M. (1997). Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell* 90: 739-751.). Migration was measured in a Boyden chamber Falk, et al., *J. Immunol.* 118:239-247 (1980) with increasing concentration of recombinant chick collapsin-1 in the bottom well and PAE cell transfectants in the upper well.
2. Aortic Ring Assay. 200 gram rats were sacrificed and the aorta is dissected between the aortic arch and kidney artery and the adipofibrotic tissue around the aorta was removed. Aortic rings were sliced at 1 mm intervals and embedded in type I collagen gels. Each ring was cultured in one well of a 48-well plate with serum-free endothelial cell medium (GIBCO). The number of microvessels were counted in each ring using a phase microscope (Miao, et al. (1997). *J. Clin. Invest.* 99: 1565-1575.).

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SEQUENCE LISTING

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TAKASHIMA, Seiji

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35 40

What is claimed:

1. A neuropilin antagonist that binds neuropilin and has VEGF antagonist activity as determined by the human umbilical vein endothelial cell (HUVEC) proliferation assay using VEGF<sub>165</sub>.
2. The neuropilin antagonist of claim 1, wherein the antagonist is an antibody that specifically inhibits binding of VEGF to a neuropilin receptor.
3. The neuropilin antagonist of claim 1, wherein the antagonist is a member of the semaphorin/collapsin family or a fragment thereof.
4. The neuropilin antagonist of claim 1, wherein the member of the semaphorin/collapsin family is collapsin-1.
5. An antibody directed against a neuropilin receptor, wherein said antibody specifically inhibits binding of VEGF to the receptor.
6. The antibody of claim 5, wherein the neuropilin is NP-1 or NP-2.
7. A method for identifying an antagonist which binds to a neuropilin, comprising exposing the neuropilin to the molecule suspected of binding thereto and determining binding of the molecule to the receptor.
8. The method of claim 7, wherein the neuropilin is NP-1 or NP-2.
9. A method of inhibiting metastasis in a patient having malignant cells which comprises:
  - (a) determining whether the patient's malignant cells express a neuropilin, and if they do adding a compound that interferes with the neuropilin.

11. The method of claim 10, wherein the compound is a antibody that specifically binds neuropilin or a neuropilin antagonist.

13. The method of claim 11, wherein the compound is a member of the semaphorin/collapsin family or a fragment thereof.

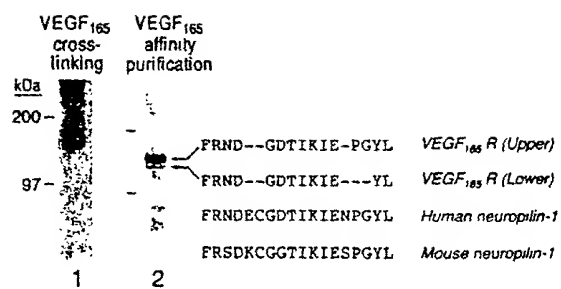
15. The method of claim 9, wherein the malignant cell is a breast or prostate cell or a melanoma.

17. Use of a member of the semaphorin/collapsin family in the preparation of a medicament for the treatment of a disease or disorder associated with VEGF.

[illegible]

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Figure 1



09580303 030000

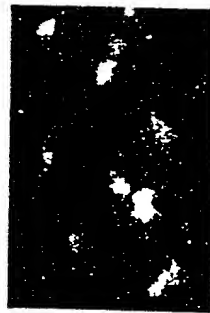


Figure 3

1	MERGLPLLCAVLALVLAPAGAF	RNDKCGDTIKIESPGYLTSPGYPHSHYHPSEKCEWLIQAPDPYQRIMIN	70	
71	FNPFDLEDLRDCKYDTVEVDGENENGHFRCKFCGKIAPPVYSSGPFIFKIFVSDYETHGAGFSIRYEI	140		
141	FKRGPESQNYHTTSPGVKSPGFPKYPNSLECTYIVFAPKMSEIILEFESFDLEPDSNPPGGMFCRYDR	210		
211	LEIWDGFPDVGPHIGRYCGQKTPGRIRSSSGILSHVFTDIAIAKEGFSANYSVLQSSVSEDFKCHZALG	280		
281	HESGEIHSQDITASSQYSTNWSAERSRLNYPENGWTPGEDSIREWIQVDLGLLRFTAVGTGGAISKETK	350		
351	KKYYVKTYYKIDVSSNGEDWITIKEGNKPVLFQGNTPDQVVVAVFVKPLITRFVRIKIPATWETGISMRFE	420		
421	VYGCKITDYPSCGMLGMVSGLISDSQITSSNQGDNRNWNPENIRLVTSRSGWALPPAPHSYINELWQIDLG	490		
491	EKIVRGIIIQGGKHRENKVFMRKFKIGYSNNGSDWKMMDDSKRKAKEFEGNNNYDTPELRTFPALSTR	560		
561	FIRIYPERATHGGLGLRMELGCEVEAPTAGPTTPNGNLVDECDQDQANCHSGTGDDFQLTGTTVLATE	630		
631	KPTVIDSTIQSEPTTYGFCNEGFGWSHKHTFCHWEHNDHVLVQLRWSVLTSKTGP IQHDTGDNFIYSQADEN	700		
701	QKGKVARLVSPVEYSQNSACHMTFYSKSHSHSVHTRVLRKYKPEEYDQLVWMAIGHQGDHWKEGRVLL	770		
771	HKSLKLYQVIFGEIGKGNLGGIAVDISINHHISQEDCAKPADLKKNPEIKIDETGSTPGYEGEGEGD	840		
841	KNISRKPGNVLKTLDPLILTI	IAMSALGVLLGAVCGVVLYCAC	WHNGMSERNLSALENYNFEVDGVKLK	910
911	KDKLNTQSTYSEA	922		

Parameter	Value	Unit
Initial temperature	25.0	°C
Final temperature	25.0	°C
Initial pressure	1.013	bar
Final pressure	1.013	bar
Initial volume	0.001	m³
Final volume	0.001	m³
Initial mass	0.001	kg
Final mass	0.001	kg
Initial density	1000	kg/m³
Final density	1000	kg/m³
Initial viscosity	0.001	Pa·s
Final viscosity	0.001	Pa·s
Initial thermal conductivity	0.6	W/m·K
Final thermal conductivity	0.6	W/m·K
Initial specific heat	4182	J/kg·K
Final specific heat	4182	J/kg·K
Initial enthalpy	4182	J/kg
Final enthalpy	4182	J/kg
Initial entropy	4182	J/kg·K
Final entropy	4182	J/kg·K
Initial internal energy	4182	J/kg
Final internal energy	4182	J/kg
Initial free energy	4182	J/kg
Final free energy	4182	J/kg
Initial Helmholtz free energy	4182	J/kg
Final Helmholtz free energy	4182	J/kg
Initial Gibbs free energy	4182	J/kg
Final Gibbs free energy	4182	J/kg
Initial chemical potential	4182	J/kg
Final chemical potential	4182	J/kg
Initial activity	1.0	
Final activity	1.0	
Initial fugacity	1.013	bar
Final fugacity	1.013	bar
Initial vapor pressure	1.013	bar
Final vapor pressure	1.013	bar
Initial saturation temperature	100	°C
Final saturation temperature	100	°C
Initial saturation pressure	1.013	bar
Final saturation pressure	1.013	bar
Initial critical temperature	374	°C
Final critical temperature	374	°C
Initial critical pressure	221	bar
Final critical pressure	221	bar
Initial critical density	322	kg/m³
Final critical density	322	kg/m³
Initial critical viscosity	0.055	Pa·s
Final critical viscosity	0.055	Pa·s
Initial critical thermal conductivity	0.12	W/m·K
Final critical thermal conductivity	0.12	W/m·K
Initial critical specific heat	1980	J/kg·K
Final critical specific heat	1980	J/kg·K
Initial critical enthalpy	1980	J/kg
Final critical enthalpy	1980	J/kg
Initial critical entropy	1980	J/kg·K
Final critical entropy	1980	J/kg·K
Initial critical internal energy	1980	J/kg
Final critical internal energy	1980	J/kg
Initial critical free energy	1980	J/kg
Final critical free energy	1980	J/kg
Initial critical Helmholtz free energy	1980	J/kg
Final critical Helmholtz free energy	1980	J/kg
Initial critical Gibbs free energy	1980	J/kg
Final critical Gibbs free energy	1980	J/kg
Initial critical chemical potential	1980	J/kg
Final critical chemical potential	1980	J/kg
Initial critical activity	1.0	
Final critical activity	1.0	
Initial critical fugacity	1.013	bar
Final critical fugacity	1.013	bar
Initial critical vapor pressure	1.013	bar
Final critical vapor pressure	1.013	bar

Figure 4

# Comparative Deduced Amino Acid Sequences of Human VEGF<sub>165</sub>R/NP and VEGF<sub>165</sub>R/NP-1

VEGF <sub>165</sub> R/NP-2	1	MDMF-PLTW-VFLALYFSRHQVRQPPPCGG-RLNSK--DA-----GY	50
VEGF <sub>165</sub> R/NP-1		MERGPLELCAV-LAL-----VLA-PA---GAFR-NOKCGDTIKIESPGY	
NP-2	51	ITSPGYPDY-FSHQNCZW-IVYAPEPNQKIVLNFNPHFEIEKHDCRYDF	100
NP-1		LTSPGYFHSZHPSEK-CEWLIQ-APDPYQRIMINFNPHFDLEDRCKYDY	
NP-2	101	IZIRDGSESAOLLGKHCGNIAPPTIISSGSMLYIKFTSDYARQGAGFSL	150
NP-1		VZVFDGENENGHFRGKFCGKIAPPPVVSAGPFLFIKPVSDYETHGAGFSI	
NP-2	151	RYEIFKTGSEDCSKNFTSPNGTIESPGFPEKYFHN-LOCTFTIL-AKPKM	200
NP-1		RYEIFKRGPE-CSQNYTTPSGVIKSPGFPEKYP-NSLECTY-IVFA-PKM	
NP-2	201	-EIIILQFLIFDLEHD--PLQVGEGD-CKYDWLDIWDGIFHVGPILIGKYCG	250
NP-1		SEIILEFESFDLEPDSNPP--G-GMFCRYDRLEIWDGFFDVGPPIGRYCG	
NP-2	251	TKTPSELRSSTGILSLTFHTDMAYAKOGESAPYYLVHQEPL-ENFQCNVP	300
NP-1		QKTPGRIRSSSGILSMVFYTDSAIAKEGFSANYG-VLQSSVSDZFKCHEA	
NP-2	301	LGMESGRIANEQISASSTYSOGRWTPQQSRDHGDDNGWTPNLOSXKEYLQ	350
NP-1		LGMESGEIHSQITASSQYSTN-WSAERSRLNYPENGWTPGEDSYREWIQ	
NP-2	351	VDL---REFLTLTAIAATQGAISRETQNGYYVRSYKLEVSTNGEDWNVYRH	400
NP-1		VDLGLDLRFVT---AVGTQGAISKETKKKYYVXTYXIDVSSNGEDWITIKE	
NP-2	401	GKNHK-V-FQAN-NCATEVVLN---KLRAPLLETRFVRIKPTWHSGLALR	450
NP-1		G-N-KPVLEQGNTHP-TDVVVAVFPPK---PLITRFVRIKPATWETGISMR	
NP-2	451	LELFGRVTDAPCSNMLGMLSGLIADSGISASSTQEYL-WSPSAARLVSS	500
NP-1		FEVYGGKITDYPSCGMLGMVSGLISDSQIT-SSNQGDNRNWPENIRLVTS	
NP-2	501	RSGWF-PRIPQAQPGG---EWLQVLDGTPKTVKGVIIQGARGGDSITAVE	550
NP-1		RSGWALP--P-A-PHSYINELQIDLGEKIVRGIIIQG--GKHRENKV-	
NP-2	551	ARAFVRKFKVSYSLNGKDWYIQDP--RTQPKLFEGNMHYDTPDIRRED	600
NP-1		---FMRKFKIGYSNNGSDWKMIMDDSKRA--KSFEGNNHYDTPDIRTF-	
NP-2	601	PIPAQYVRV---YPERWSPA--GI-GMRLEVLCGDWTDKPTVE--TLGP	650
NP-1		P--ALSTRFIRIYPER---ATHGGGLRLMELLGCE-----VEAPTAGP	
NP-2	651	TVKSEETTPYPTSEATECGE---NC-SFE-DDKDQLQ-----L---P-	700
NP-1		T-----T--PNGNLVD--ECDDQANCHSGTGDDFQLTGTTVLATEKPT	
NP-2	701	---S-----GFNCIFD-----FLEEFPGWMYD-BA--KW--LRIT	750
NP-1		VIDSTIQSEFPTYGFNCEFGWGSHTF---CHWEHDNHVQLKNSVL-T-	
NP-2	751	WASSSSSPN-DRTEPDORHPLRLQSDS-QREGQYARLISPPVHLPRSPVCM	800
NP-1		--SKTGPIQDHTG-DG-NFIYSQADENQK-GKVARLVSPVVYSQNSAHCM	
NP-2	801	EFQYQATG---G--RGVAL--QVVREASQESKLLWV-IREDQGGGWKHGR	850
NP-1		TFWYHMSGSHVGTLR-VKLRYQKPEEYDQ---LVWMAIGH-QGDHWKEGR	
NP-2	851	IILP-SYDMYQ-IVFEGVIGKGRSGEIAIDDIRI---STOVPLENCME	900
NP-1		VLLHKSLLK-YQVI-FEGEIGKGNLGGIAVDISINNHSIQ---EDCAK	
NP-2	901	PISAFAGENFKVDIPEIHERS-G---YEDEIDDEYVDWNSSSSATSGS	950
NP-1		P--ADLGR--KN--PEIKIDETGSTPGYEGEG--EG--DK-NISRPK-GN	
NP-2	951	GAPSTDREKSWLYTLDPIILITIAMSSLGVLGATCAGLLLYCTCSYSGI	1000
NP-1		VL----K-----TLDPIILITIAMSSALGVLLGAVC-GVVLYCACWENGM	
NP-2	1001	SSR--SCITLENYHFELYDG--LKHKVVNNHQQCCSEA	1038
NP-1		SERNLSA--LENYHFELVDGVYKLG-KDRLENTQSTYSERL	

Figure 5

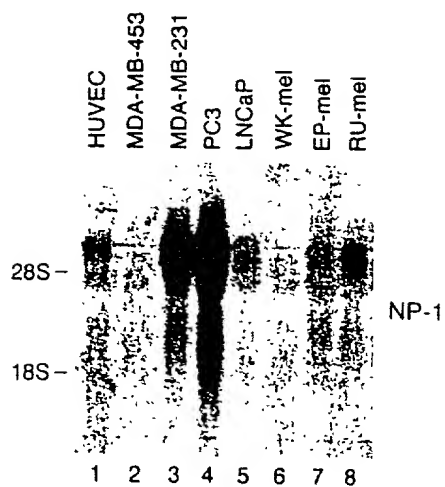


Figure 6

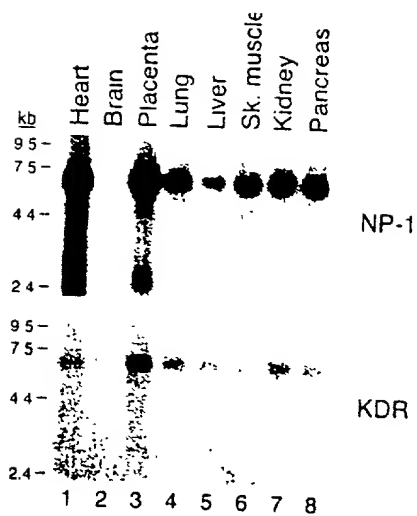


Figure 7A

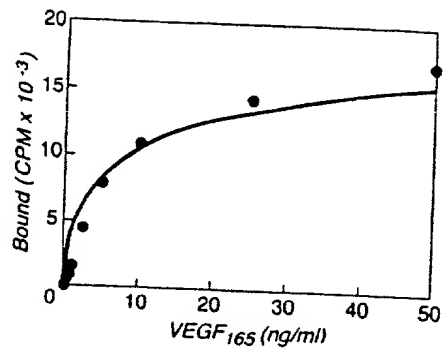


Figure 7B

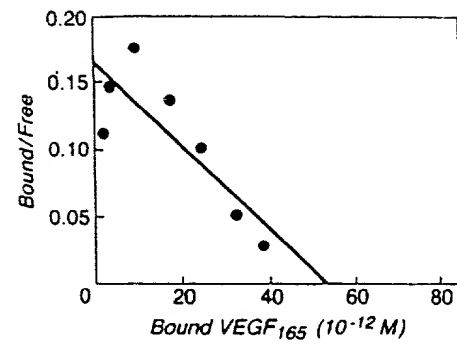


Figure 8

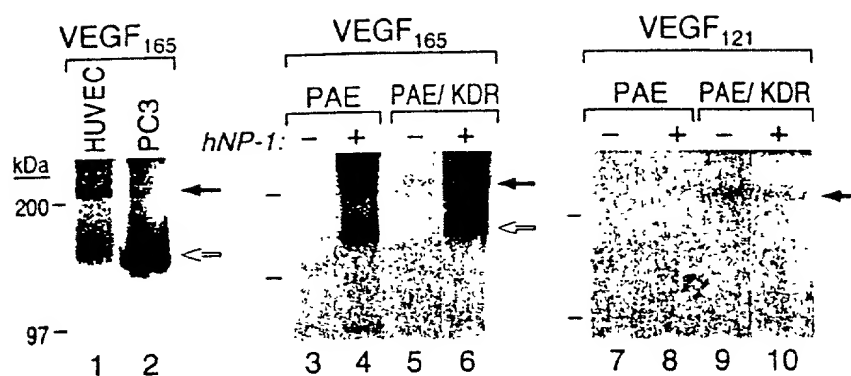


Figure 9

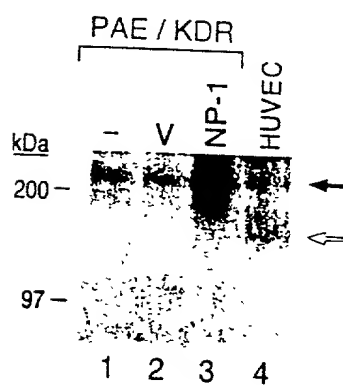


Figure 10



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Figure 11A  
KDR alone

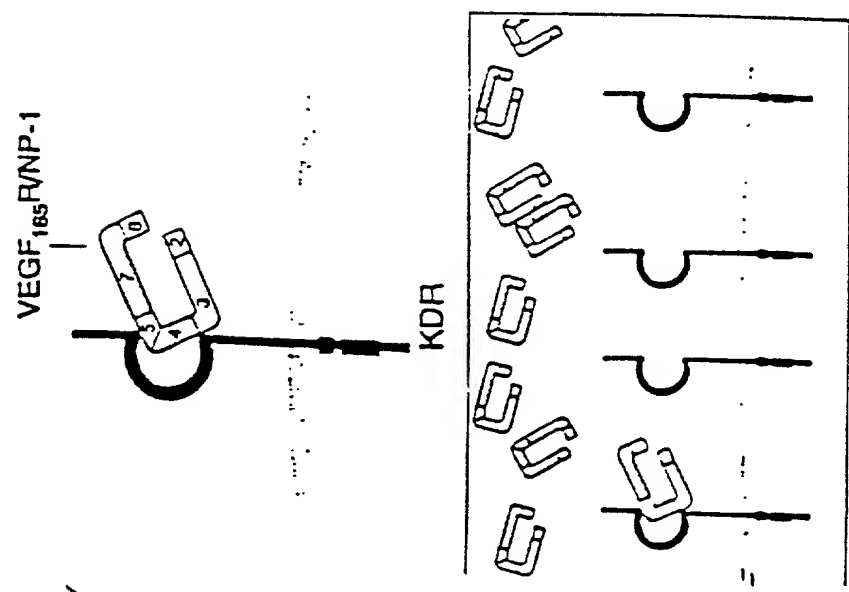


Figure 11B  
KDR + VEGF<sub>165</sub>R/NP-1

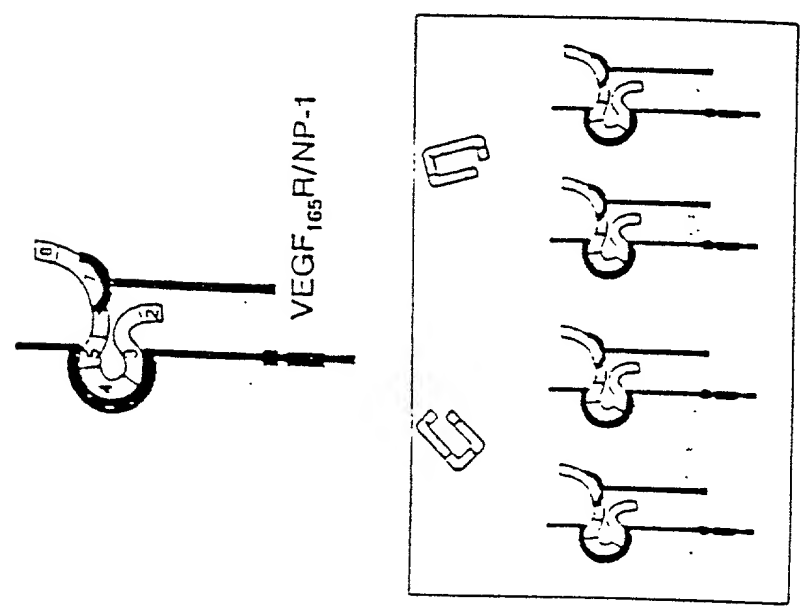
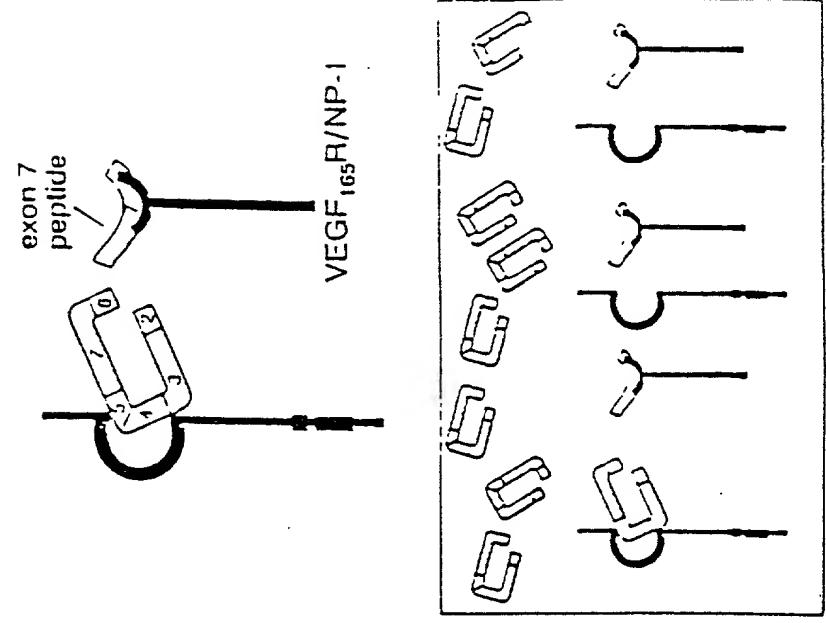


Figure 11C  
KDR + VEGF<sub>165</sub>R/NP-1  
+ Exon 7



[illegible]

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CEWVYAPEPNCKIVLNFNPHFEIEKHDCCKYDFEIRDGDOSESADLLGKHCGNIAPP  
TISSGSMLYIKFTSDYARQGAGFSLRYEIFKTGSEDCSKNFTSPNGTIESFGFPEK  
YPHNLDCTFTILAKPKMEIILQFLIFDLEHDPLOVGEEDCKYDWLDIWDGIPHVGPL  
IGKYCGTKTPSELRSSTGILSLTFHTDMAVAKDGFSARYYL VHQEPLNFQCNVP  
LGMESGRIANEQISASSTYS DGRWTPQQSRLHGDDNGWTPNLDSNKEYLQVDLR  
FLTMLTAIATQGAISRETQNGYYVKSYLEVSTNGEDWMVYRHGKNHKVFOANN  
DATEVVLNKLHAPLLTRFVRIRPQTWHSGIALRLELFGCRVTDAPCSNMLGMLS  
GLIADSQISASSTOEYLWSPSAARLVSSRS GWFPRIPOAQFGEEWLQVDLGTPK  
TVKGVIIQGARGGDSITAVEARAFVRKFKVSYSLNGKDWEYIQDPRTQQPKLFEG  
NMHYDTPDIRRFDPIPAQYVRVYPERWSPAGIGMPLEVLGODWTD SKPTVETLG  
PTVKSEETTPYPTEEEATECGENC SFEDDKDLQLPSGFNCNFD FLEEPCGWMYD  
HAKWLRTTWASSSSPNDRTPDDRNLRLQSDSQREGQYARLSPPVHLPRSPV  
CMEFQYQATGGRGVALQVWREASQESKLLWVIREDOGGEWKHGRILPSYDMEYQ  
IVFEGVIGKGRSGEIAIDDIRISTDVPLENCMEPI SAFAGENFKVDIPEIHEREGYED  
EIDDEYEVDWSNSSSSATSGSGAPSTDKEKSWLYTLDPILITIIAMSSLGVL LGAT  
CAGLLLYOTCSYSGLSRSSCTTLENYNFELYDGLKHVKVMNHOKCCSEA\*

Figure 13

gaattcggca	cgaggggaaa	ataaaagaga	gaaaaacaca	aagattttaa	caagaaacct	60
acgaacccag	ctctggaaa	agccaccttc	tccaaaatgg	atatgtttcc	tctcacctgg	120
gttttcttag	ccctctactt	ttcaagacac	caagtggag	gccaaccaga	cccaccgtgc	180
ggaggtcggt	tgaattccaa	agatgctggc	tatatcacct	ctcccgggta	cccccaggac	240
tacccctccc	accagaactg	cgagtggatt	gtttacgccc	ccgaacccaa	ccagaagatt	300
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gagattcggg	atggggacag	tgaatccgca	gacctcctgg	gcaaacactg	tgggaacatc	420
gccccgcccc	ccatcatctc	ctcgggctcc	atgctctaca	tcaagttcac	ctccgactac	480
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tgctcaaaaa	acttcacaag	ccccaacggg	accatcgaat	ctcctggggt	tcttgagaag	600
tatccacaca	acttggaactg	cacctttacc	atcctggcca	aaccaagat	ggagatcatc	660
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tttcacacgg	acatggcggg	ggccaaggat	ggcttctctg	cgcttacta	cctgggtccac	900
caagagccac	tagagaactt	tcagtgcatt	gttccctctg	gcatggagtc	tgcccgatt	960
gctaataaac	agatcagtg	ctcatctacc	tactctgatg	ggaggtggac	ccctcaacaa	1020
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gatcattatt	ctccgcttct	tctttctaat	caacacttga	aaagcaaagt	gtcttttcag	3360
cctttccatc	tttacaataa	aaactcaaaa	aagctgtcca	gctt		3404

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Figure 1 consists of 12 bar charts, labeled (a) through (l), each representing a different demographic or attitudinal category. The categories are: (a) Age, (b) Sex, (c) Education, (d) Employment, (e) Income, (f) Health, (g) Social, (h) Family, (i) Religion, (j) Culture, (k) Values, and (l) Attitudes. Each chart has a y-axis labeled 'Percent' ranging from 0 to 100. The x-axis for each chart lists four groups: Control, Mild, Moderate, and Severe. The bars are color-coded: white for Control, light gray for Mild, medium gray for Moderate, and dark gray for Severe. The data shows varying distributions across groups. For example, in (a) Age, the Control group has the highest percentage (around 75%), while in (f) Health, the Severe group has the highest percentage (around 75%).

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Figure 1 consists of 15 bar charts, labeled (a) through (p), each representing a different demographic or socio-economic parameter. The x-axis for each chart is divided into two categories: 'PLHIV' (People Living With HIV/AIDS) and 'General Population'. The y-axis represents the percentage of the total population for each category. The parameters are as follows:

- (a) Age: 15-24, 25-34, 35-44, 45-54, 55-64, 65-74, 75+.
- (b) Sex: Male, Female.
- (c) Education: No formal education, Primary, Secondary, Tertiary.
- (d) Occupation: Unemployed, Informal sector, Formal sector, Self-employed.
- (e) Marital status: Single, Married, Divorced, Widowed.
- (f) Religion: Christianity, Islam, Hinduism, Buddhism, Other.
- (g) Ethnicity: African, Asian, European, Other.
- (h) Income: Low, Middle, High.
- (i) Health status: Good, Fair, Poor.
- (j) Access to health services: Yes, No.
- (k) Knowledge of HIV/AIDS: Yes, No.
- (l) Attitudes towards people with HIV/AIDS: Accepting, Not accepting.
- (m) Stigma: High, Low.
- (n) Discrimination: Yes, No.
- (o) Support from family and community: Yes, No.
- (p) Access to antiretroviral therapy: Yes, No.

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2623			2632			2641			2650			2659			2668		
ATT	GCT	GTG	GAT	GAC	ATT	AGT	ATT	AAC	AAC	CAC	ATT	TCA	CAA	GAA	GAT	TGT	GCA
I	A	V	D	D	T	S	I	N	N	H	I	S	Q	E	D	C	A
2677			2686			2695			2704			2713			2722		
AAA	CCA	GCA	GAC	CTG	GAT	AAA	AAG	AAC	CCA	GAA	ATT	AAA	ATT	GAT	GAA	ACA	GGG
K	P	A	D	L	D	K	K	N	P	E	I	K	I	D	E	T	G
2731			2740			2749			2758			2767			2776		
AGC	ACG	CCA	GGA	TAC	GAA	GGT	GAA	GGA	GAA	GGT	GAC	AAG	AAC	ATC	TCC	AGG	AAG
S	T	P	G	Y	E	G	E	G	E	G	D	K	N	I	S	R	K
2785			2794			2803			2812			2821			2830		
CCA	GGC	AAT	GTG	TTG	AAG	ACC	TTA	GAT	CCC	ATC	CTC	ATC	ACC	ATC	ATA	GCC	ATG
P	G	N	V	L	K	T	L	D	P	L	I	I	T	I	I	A	M
2839			2848			2857			2866			2875			2884		
AGT	GCC	CTG	GGG	GTC	CTC	CTG	GGG	GCT	GTC	TGT	GGG	GTC	GTG	CTG	TAC	TGT	GCC
S	A	L	G	V	L	L	G	A	V	C	G	V	V	L	Y	C	A
2893			2902			2911			2920			2929			2938		
TGT	TGG	CAT	AAT	GGG	ATG	TCA	GAA	AGA	AAC	TTG	TCT	GCC	CTG	GAG	AAC	TAT	AAC
C <sub>TM</sub>	W	L	C <sub>N</sub>	G	M	S	E	R	N	L	S	A	L	E	N	Y	N
2947			2956			2965			2974			2983			2992		
TTT	GAA	CTT	GTG	GAT	GGT	GTG	AAG	TTG	AAA	AAA	GAC	AAA	CTG	AAT	ACA	CAG	AGT
F	F	L	V	D	G	V	K	L	K	K	D	K	L	N	T	Q	S
3001			3010														
ACT	TAT	TCG	GAG	GCA	TGA	3' (SEQ ID NO: 1)											
T	Y	S	E	A		(SEQ ID NO: 4)											
			cyto			2											

Figure 15A

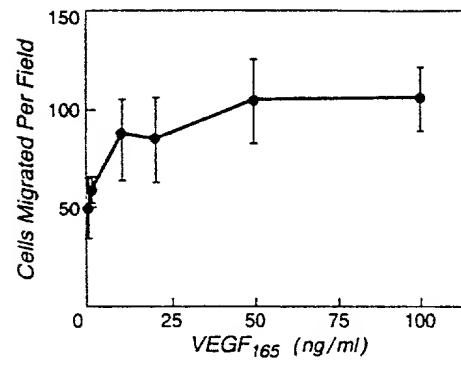


Figure 15B

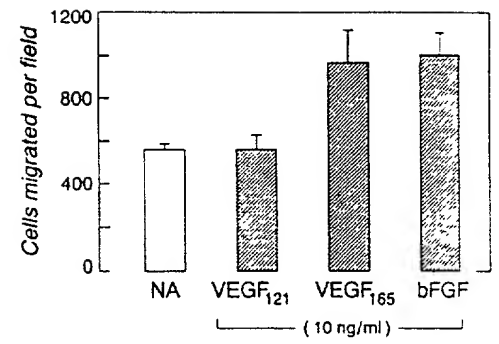


Figure 16A

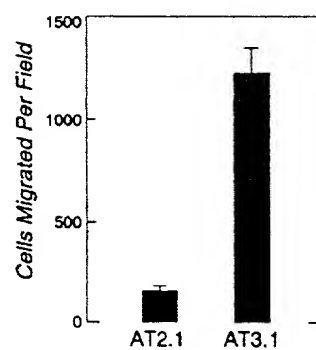


Figure 16B

B

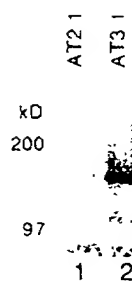


Figure 16C

C

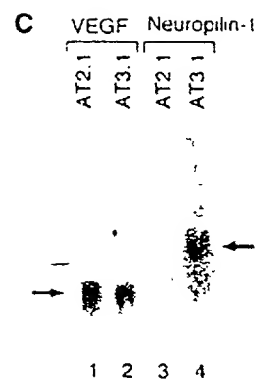


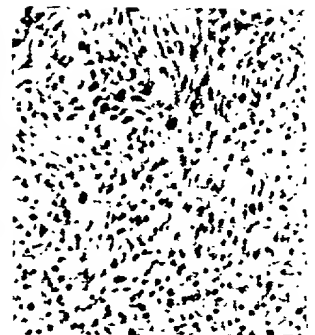
Figure 17A



Figure 17B



Figure 17C

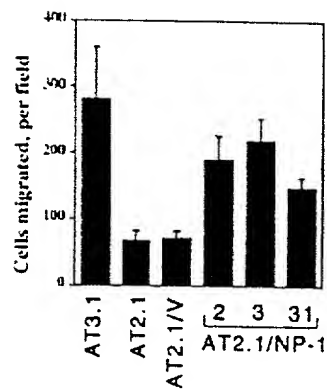


000050" E0808560

Figure 18A



Figure 18B



NP-1

NP-2

$\beta$ -actin

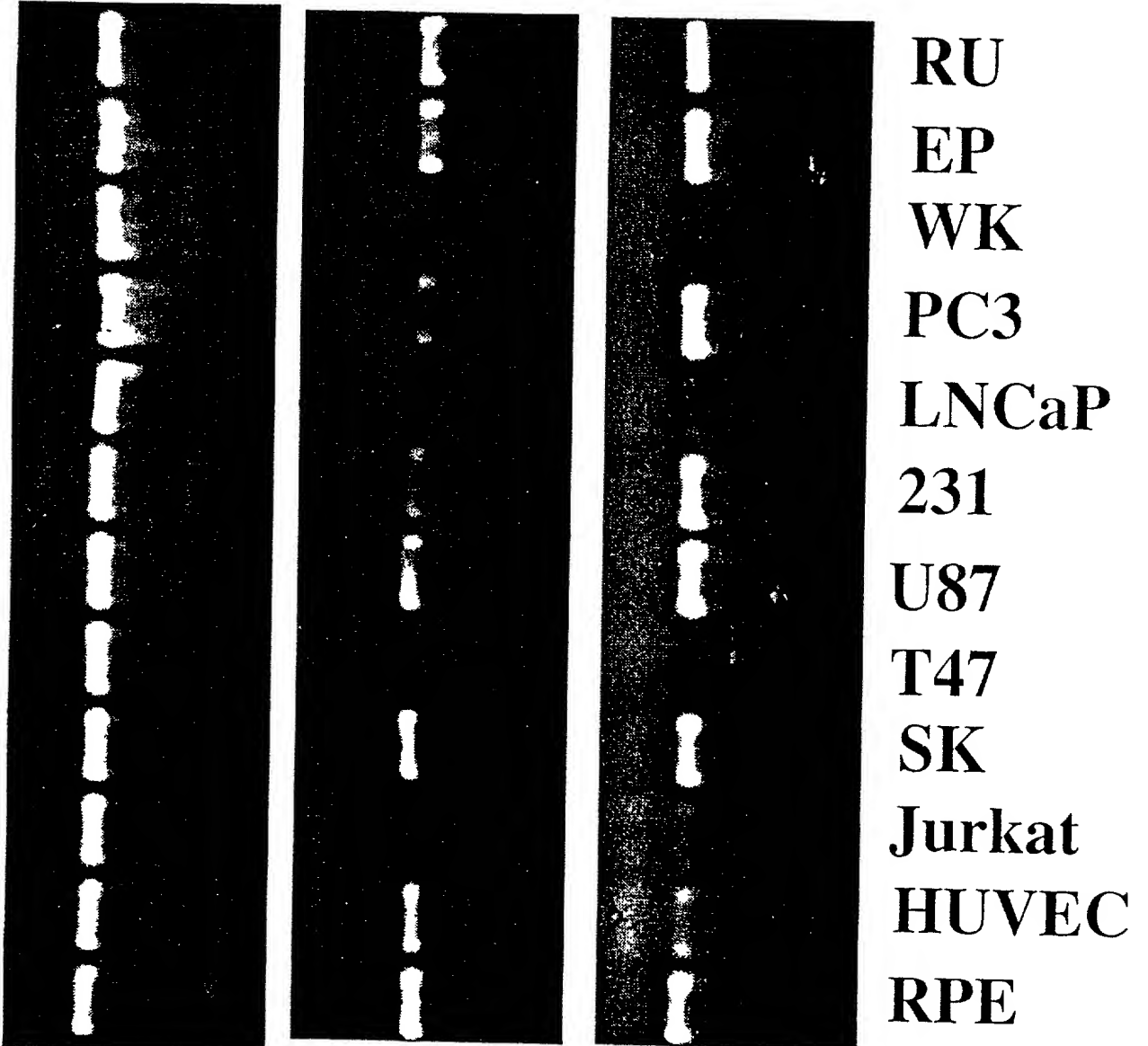


Figure 20

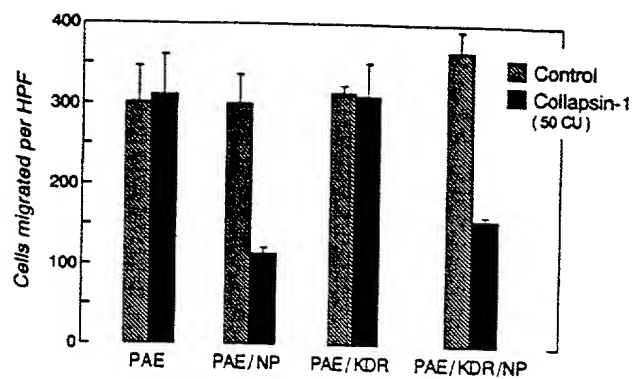


Figure 21A

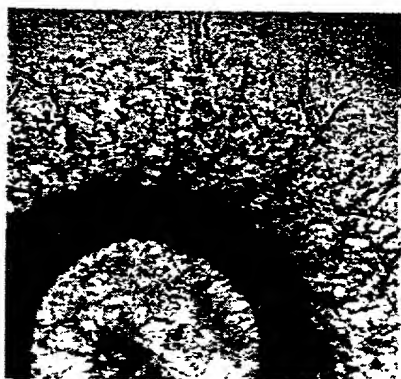


Figure 21B

